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13. ABSTRACT (Maximum 200 Words)

The purpose of this Career Development Award are to provide the salary support to foster the career development of the applicant and to study the Estrogen receptor (ER) target gene transcriptional activation in estrogen target organs. The scope of the research is to create an in vivo ER transactivation model to study the molecular mechanisms of estrogen, anti-estrogen, and growth factor action associated with ER target gene transcriptional activation in mammary gland, uterus, and bone. The major training accomplishment of this CDA is the promotions from non-tenure track Research Assistant Professor to tenure track Assistant Professor and then to Associate Professor, awards of RO1 and other research grants, invitation to chair symposia at international meetings, and invitation to various study sections. The applicant has established her own active research program in the area of breast cancer research because of the support from this CDA. The major research accomplishments include publications in major journals, presentations of data at international meetings, and several manuscripts in preparation. The major research findings include the successful establishment of an in vivo model of ER transactivation using adenovirus approach and the discovery of the segregation of steroid receptor coactivator -1 from Erα in normal mammary epithelial cells.

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Introduction

The purposes of this Career Development Award are to provide the salary support to foster the career development of the PI and to study the Estrogen receptor (ER) target gene transcriptional activation in estrogen target organs. The scope of the research is to study the molecular mechanisms of estrogen, anti-estrogen, and growth factor action associated with ER target gene transcriptional activation in mammary gland, uterus, and bone. Evaluation of the expression and activation of steroid receptor coactivators and corepressors is also the subject of this project.

Body

Task 1: Completed. We have successfully delivered the adenovirus Ad-CMV-βgal into mammary gland, uterus, and bone (1,2).

Task 2: Completed. We have successfully constructed several adenoviral vectors (1). Ad-CMV-GFP, Ad-CMV-βgal and Ad-ERE-βgal . Since we have good results generated from these reporter viruses. We did not construct the Ad-CMV-CAT.

Task 3: Completed. We have successfully generated the recombinant adenoviruses.

Task 4: Completed. We have successfully purified and titrated the recombinant adenoviruses.

Task 5: Completed. The adenovirus reporters have been tested in tissue culture cells with their infectivity and responsiveness to estrogen (1).

Task 6: Completed. Introduction of adenovirus into mammary gland by reporter virus did not reveal gross changes of mammary gland structure.

Task 7: Completed. We have adopted a more sensitive method to detect the proliferation status of mammary cells using anti-PCNA or Ki67 antibody and have found that only a portion of the mammary cells can be induced to proliferate in mammary or uterus cells by estrogen treatment (2).

Task 8: Completed. We have found that progesterone receptor (PR) induction correlated with the activation of Ad-ERE-βgal reporter (1,2, 3).

Task 9: Completed. We have delivered the reporter virus into uterus, bone and mammary gland. Estrogen was able to activate Ad-ERE-βgal in mammary gland and antiestrogen was able to abolish the estrogen-induced Ad-ERE-βgal activity (1). The ability of estrogen to induce ER target gene activation is reconstituted *in situ* in mammary gland. Due to the high cost of growth factor pellets and the budget limitation, we did not pursue the study looking at the effect of growth factor on ERE reporter activity *in situ*. Ad-CMV-βgal infected uterine epithelium and bone marrow cells with high infectivity (2, 4). However, Ad-ERE-βgal infusion resulted in high background activity in uterine epithelium bone marrow cells. Therefore, the attempt to reconstitute the ER-dependent gene activation *in situ* in uterus or bone has not been successful. We speculate that the low concentration of circulating estrogen is able to activate Ad-ERE-βgal reporter activity in uterus and bone, as compared to mammary epithelium.

Task 10: Completed. We have transfected steroid receptor coactivators and corepressors into breast cancer cells and examine their effects on ERE reporter activity. We found that steroid receptor coactivators, SRC-1, TIF2 and AIB1 were able to activate ERE reporter activity. On the other hand, steroid receptor corepressors SMRT or N-CoR was able to inhibit the ERE reporter activity in MCF-7 cells. We also examined the expression of SRC-1, TIF2, and AIB1 in human breast tumors and in normal breast cells. We have generated our own anti-AIB1 antibody in order to evaluate the expression of AIB1 in breast cancer cells. We found that AIB1 was overexpressed not only in ERα positive breast tumors, but also in ERα negative breast tumors (5). Interestingly, we found that SRC-1 was segregated from ERα and progesterone receptor (PR) in normal mammary epithelium and that SRC-1 was expressed in a subset of mammary epithelial cells. More importantly, SRC-1 was not necessary for ERα-mediated induction of PR in mammary epithelial cells and was also not sufficient for PR induction in mammary stromal cells expressing both ERα and SRC-1 (3).

Task 11: Completed. We have successfully constructed the adenovirus expressing SRC-1 GRIP1, and AIB1. Construction of Ad-CMV-SMRT has not been successful.

Task 12: Initiated. We have completed testing adenoviruses constructed in tissue culture cells. However, the testing of these adenoviruses expressing steroid receptor coactivators in estrogen target organs has just been initiated.

Key Training and Research Accomplishments

Training:

- Promotions from non-tenure track Research Assistant Professor to tenure track Assistant Professor at University of Virginia and then to Associate Professor at Indiana University.
- Awards of RO1 and other extramural and institutional research grants.
- Invitation to chair symposia at international meetings such as American Association for Cancer Research and The Endocrine Society.
- Invitation to various study sections such as NIH, American Cancer Society, and DOD.
- Establishment of an active research program in the area of breast cancer research and hormone action.

Research:

- We have successfully established the *in vivo* ER transactivation model combining intraductal injection and adenovirus approach in rat mammary gland.
- We have successfully delivered the adenovirus reporter into the rat uterus.
- We found that SRC-1 was segregated from ERα and PR in mammary epithelium.
- We have demonstrated the spatial and temporal expression of SRC-1 in mammary epithelium.
- We found that SRC-1 was not necessary for ERα-mediated induction of PR in mammary epithelial cells and was also not sufficient for PR induction in mammary stromal cells expressing both ERα and SRC-1.
- We found that AIB1 was overexpressed not only in ERα positive breast tumors, but also in ERα negative breast tumors.

Reportable Outcomes

Manuscripts:

1. **Jeng, M.-H.,** Kao, C., Sivaraman, L., Krnacik, S., Chung, L. W. K., Medina, D., Conneely, O. M., and O'Malley, B. W. Reconstitution of estrogen dependent transcriptional activation of an adenoviral target gene in select regions of the rat mammary gland. Endocrinology 139(6): 2916-2925, 1998.

2. Shim, W.-S., DiRenzo, J., DeCaprio, J. A., Santen, R. J., Brown, M. J., **Jeng, M.-H.** Segregation of steroid receptor coactivator-1 from steroid receptors in mammary epithelium. Proceedings of the National Academy of Sciences 96(1): 208-213, 1999.

3. **Jeng, M.-H.** and Kao, C. Desensitization of estrogen receptor transactivation function in normal rat mammary epithelium *in situ*. (in preparation).

4. Shim, W.-S. and **Jeng, M.-H.** Tissue-specific expression and regulation of steroid receptor coactivators (in preparation).

Abstracts:

1. Shim, W.-S., Eischeid, A. C., and **Jeng, M.-H.** Decreased transactivation function of estrogen receptors *in situ* during mammary gland development. 18th Annual American Cancer Society Seminar of Cancer Researchers at Virginia, March, 1998.
2. **Jeng, M.-H.,** Shim, W.-S., and Eischeid, A. C. Decreased transactivation function of estrogen receptors *in situ* during mammary gland development. 80th Annual Meeting of the Endocrine Society Meeting,June, 1998.
3. Shim, W.-S., Santen, R. J., Brown, M., and **Jeng, M.-H.** Discrete Expression Pattern of Steroid Receptor Coactivators-1 (SRC-1) and Estrogen Receptor α (ERα) in Estrogen-Responsive mammary Gland and Uterus. 19th American Cancer Society Seminar of Cancer Researchers at Virginia, March, 1999.
4. **Jeng, M.-H.,** Shim, W.-S., Santen, R. J., Kao, C., Brown, M., Tsai, M.-J., and O'Malley, B. W. Functions of Steroid Receptor Coactivators in Breast Tissue. 19th American Cancer Society Seminar of Cancer Researchers at Virginia, March, 1999.
5. Shim, W.-S., Santen, R. J., Brown, M., and **Jeng, M.-H.** Discrete Expression Pattern of Steroid Receptor Coactivator-1 (SRC-1) and Estrogen Receptor α (ERα) in Estrogen Responsive Mammary Gland and Uterus. 90th Annual Meeting of the American Association for Cancer Research, April, 1999.
6. **Jeng, M.-H.** and Shim, W.-S. Discrete Expression Pattern of Steroid Receptor Coactivators in Mammary Gland. Gordon Research Conference on Mammary Gland Biology. Henniker, New Hampshire, June, 1999.
7. **Jeng, M.-H.** and Shim, W.-S. Steroid Receptor Coactivators in Normal Mammary Gland. 2nd Era of Hope Meeting, June, 2000.
8. Zhang, Q.-H., Chang, L.-Y., Goulet, R.J., Edwards, D.P., Jeng, M.-H. Overexpression of SRC-1 Protein in Human Breast Tumors. 92nd Annual Meeting of the American Association for Cancer Research, March, 2001.
9. Shim, W.-S., Turner, M.A., Santen, R.J., **Jeng, M.-H.** Up-regulation of SRC-1

Protein and Increase of ER *in situ* Transactivation Function by pregnancy Hormones. 83rd Annual Meeting of the Endocrine Society, June, 2001.

10. Zhang, Q.-H., Chang, L.-Y., Vieth, E., Stallcup, M.R., Edwards, D.P., Cheng, L., Goulet, R.J., and **Jeng, M.-H.** Over-expression of Several Nuclear Receptor Coactivator Proteins in Human Breast Carcinoma. 83rd Annual Meeting of the Endocrine Society, June, 2001.
11. **Jeng, M.-H.,** Zhang, Q.-H., Long, X., Goulet, R., Sledge, G., Li, L., and Quilliam L. Functions of Estrogen Receptor Coregulators in Breast Cancer. 3rd DOD Era of Hope Meeting, September, 2002.

Presentations:

1. Shim, W.-S., Santen, R. J., Brown, M., and **Jeng, M.-H.** Discrete Expression Pattern of Steroid Receptor Coactivator-1 (SRC-1) and Estrogen Receptor α (ERα) in Estrogen Responsive Mammary Gland and Uterus. 90th Annual Meeting of the American Association for Cancer Research, April, 1999. Oral presentation.
2. Zhang, Q.-H., Chang, L.-Y., Goulet, R.J., Edwards, D.P., Jeng, M.-H. Overexpression of SRC-1 Protein in Human Breast Tumors. 92nd Annual Meeting of the American Association for Cancer Research, March, 2001. Oral presentation.
3. Shim, W.-S., Turner, M.A., Santen, R.J., **Jeng, M.-H.** Up-regulation of SRC-1 Protein and Increase of ER *in situ* Transactivation Function by pregnancy Hormones. 83rd Annual Meeting of the Endocrine Society, June, 2001. Oral presentation.

Development of research reagents:

1. Adenoviruses: Ad-CMV-βgal, Ad-ERE-βgal, Ad-CMV-βgal, Ad-CMV-SRC-1, Ad-CMV-GRIP1, and Ad-CMV-AIB1.
2. Ploryclonal antibody against AIB1.

Awards of research funding:

a). Title: Functions of Estrogen Receptor Coactivators in Breast Tissues
Role and Time Commitment: PI, 30%
Supporting Agency: NIH (NCI), 1-RO1-CA82565
Duration and Level of Funding: 7/8/99-5/31/04; current year direct costs: $145,828; total direct costs: $704,516; total costs: $1,032,316
This project received a priority score of 115 and a percentile of 0.3.

b). Title: Roles of Steroid Receptor Coactivators in Breast Cancer
Role and Time Commitment on Project: PI, 10%
Supporting Agency: U.S. Army Department of Defense, Breast Cancer Research Program, BC980498
Duration and Level of Funding: 8/1/99-1/31/04; first year direct costs: $69,518; total direct costs: $209,982; total costs: $309,477
This project received a priority score of 1.2 and a percentile of 98 (equivalent to NIH priority score of 120 and percentile of 2).

c). Title: Tissue Specific Gene Therapy for Pulmonary Metastasis

Role and Time Commitment on Project: Co-investigator, 10% (PI: Chinghai Kao, Ph.D.)
Supporting Agency: NIH/NCI
Duration and Level of Funding: 12/1/00-11/30/03; first year direct costs: $195,000

d). Title: Therapeutic Response Evaluation of A Novel Retargeting Strategy for Breast Cancer Gene Therapy Using PET Imaging Technique
Role and Time Commitment on Project: PI: 3% effort
Supporting Agency: NCI ICMIC P20 Planning Grant , pilot project
Duration and Level of Funding: 04/02-04/04, direct costs: $84,000

e). Title: Identification of AIB1 Downstream Target Genes in Human Breast Cancer Cells using Genomic Approach
Role: PI
Supporting Agency: IU School of Medicine Biomedical Research Committee
Duration and Level of Funding: 08/02-10/03, direct costs: $9,600

f). Title: Steroid Receptor Coactivator CARM1 in Breast Development and Carcinogenesis
Role: PI
Supporting Agency: IU School of Medicine Biomedical Research Committee
Duration and Level of Funding: 08/03-7/04, direct costs: $10,000

g). Title: Regulation of Estrogen Receptor Function by Ubiquitin-Like NEDD8 Pathway, #RSG TBE-104125
Role and Time Commitment on Project: Co-PI, 5% effort (PI: Kenneth Nephew, Ph.D.)
Supporting Agency: American Cancer Society
Duration and level of Funding: 07/01/02-06/30/06, direct costs: $751,851; indirect costs: $187,963; total costs: $939,814

h). Title CARM1 in Mitogenesis and Breast Cancer
Role and Time Commitment on Project: PI, 3% effort
Supporting Agency: Peachy Fund, IU Cancer Center
Duration and Level of Funding: 07/01/00-06/30/02, direct costs: $20,000

i). Title: Estrogen Receptor Coactivators in Mitogenesis of Breast Tissues
Role and Time Commitment of Project: PI, 10%
Supporting Agency: University of Virginia Cancer Cancer Support Grant and NIH P30CA44579, a pilot project
Duration and Level of Funding: 12/1/98-7/31/99; current/total direct costs: $29,211; total costs: $43,232

j). Title: Steroid Receptor Coactivators in Estrogen Responsive Tissues
Role and Time Commitment of Project: PI, 10%
Supporting Agency: University of Virginia School of Medicine Research and Development Committee
Duration and Level of Funding: 2/1/99-1/31/00; current/total direct costs: $15,000;

total costs: $15,000

k). Title: Functions of Estrogen Receptor Coactivators in Breast Tissues
Role and Time Commitment: PI, 40%
Supporting Agency: American Cancer Society, RPG-TBE-98273
Duration and Level of Funding: 7/1/99-6/30/02; first year direct costs: $106,108; total direct costs: $356,237; total costs: $445,296
This award was declined.
This project received a top 10 percentile.

Employments:

1. Promotion from non-tenure track Research Assistant Professor to tenure track Assistant Professor at University of Virginia.
2. Promotion to Associate Professor at Indiana University.
3. Named as the Andrew and Peggy Thomson Investigator in Breast Cancer Research at Indiana University.

Invitations to Chair Symposia and to participate in Study Sections:

Study Sections: NIH, American Cancer Society, and DOD study sections

Symposia:

1. Program Committee , Receptors and Signal Transduction Section of the Endocrinology: Molecular and Preclinical Subcommittee, American Association for Cancer Research Annual Meeting
2. Chairperson, Poster Discussion Session, Endocrinology: Molecular and Preclinical3: Steroid Hormones and Growth Factors, American Association for Cancer Research Annual Meeting
3. Symposium Chair, Hormone-Dependent Breast and Prostate Cancer, The Endocrine Society Annual Meeting

Conclusions

During the funding period of this Career Development Award, the PI has major training and research accomplishments. She has established an active breast cancer research program, has been funded by several extramural grants, has been promoted from non-tenure track to tenure track then to Associate Professor position, has published in major journals, has several manuscripts in preparation, and has been invited to chair symposia and to participate in study sections. This CDA has provided the opportunity for a junior investigator and has paved a solid foundation to allow the PI to pursue a career in breast cancer research. She has established a unique animal model to study the molecular mechanisms of hormones and has found that SRC-1 was segregated from ERα in mammary epithelium. It is hopeful that one day she can make a major contribution to breast cancer research.

References

1. Jeng, M.-H., Kao, C., Sivaraman, L., Krnacik, S., Chung, L. W. K., Medina, D.,

Conneely, O. M., and O'Malley, B. W. Reconstitution of estrogen dependent transcriptional activation of an adenoviral target gene in select regions of the rat mammary gland. Endocrinology 139(6): 2916-2925, 1998.
2. See annual report dated August 1998.
3. Shim, W.-S., DiRenzo, J., DeCaprio, J. A., Santen, R. J., Brown, M. J., Jeng, M.-H. Segregation of steroid receptor coactivator-1 from steroid receptors in mammary epithelium. Proceedings of the National Academy of Sciences 96(1): 208-213, 1999.
4. See annual report dated August 1999.
5. Zhang, Q.-H., Chang, L.-Y., Vieth, E., Stallcup, M.R., Edwards, D.P., Cheng, L., Goulet, R.J., and Jeng, M.-H. Over-expression of Several Nuclear Receptor Coactivator Proteins in Human Breast Carcinoma. 83rd Annual Meeting of the Endocrine Society, June, 2001.

Appendices

Manuscripts and abstracts.

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 208–213, January 1999
Medical Sciences

Segregation of steroid receptor coactivator-1 from steroid receptors in mammary epithelium

WOO-SHIN SHIM*, JAMES DIRENZO†, JAMES A. DECAPRIO†, RICHARD J. SANTEN*, MYLES BROWN†, AND MEEI-HUEY JENG*‡

*Department of Internal Medicine, Division of Hematology/Oncology, University of Virginia Health Sciences Center, Charlottesville, VA 22908; and †Department of Adult Oncology, Dana–Farber Cancer Institute, Harvard Medical School, Boston, MA 02115

Communicated by Robert A. Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA, October 26, 1998 (received for review August 7, 1998)

ABSTRACT **Steroid receptor coactivator-1 (SRC-1) family members interact with steroid receptors, including estrogen receptor α (ERα) and progesterone receptor (PR), to enhance ligand-dependent transcription. However, the expression of ERα and SRC-1 was found to be segregated in distinct subsets of cells within the epithelium of the estrogen-responsive rat mammary gland. This finding was in contrast to the finding for the stroma, where significant numbers of cells coexpressed ERα and SRC-1. Treatment of animals with estrogen induced PR expression in the ERα-expressing mammary epithelial cells in the absence of detectable SRC-1 and did not affect the segregated pattern of SRC-1 and ERα expression. PR was neither expressed nor induced by estrogen treatment in stroma, despite the coexpression of ERα and SRC-1. These results suggest that SRC-1 is not necessary for ERα-mediated induction of PR in mammary epithelial cells and is also not sufficient for PR induction in stromal cells expressing both ERα and SRC-1. Furthermore, the expression of SRC-1 in a subpopulation of mammary epithelial cells distinct from those expressing ERα or PR raises the possibility that SRC-1 has cell type-specific functions other than simply to act as coactivator for ERα or PR in the mammary epithelium.**

Ovarian steroids play a critical role in mammary gland development, acting through specific receptors expressed in target cells. When steroid receptors become bound to hormones, the receptors undergo a conformational change, bind to their cognate DNA response elements in target genes, recruit coactivators and general transcription factors, and subsequently activate target gene expression. Several coactivators have been cloned, and many of these coactivator proteins were initially identified biochemically as nuclear receptor-interacting proteins of approximately 160 kDa based on their ability to interact with agonist-bound estrogen receptor α (ERα) (1, 2). To date, three distinct but related p160 family members have been identified, with each family member having a number of splice variants. This family includes steroid receptor coactivator-1 (SRC-1) [also designated nuclear receptor coactivator 1 (NCoA-1)], glucocorticoid receptor-interacting protein 1 (GRIP1) [also designated nuclear receptor coactivator 2 (NCoA-2), transcriptional intermediary factor 2 (TIF2), or steroid receptor coactivator-2 (SRC-2)], and p300/cAMP response element-binding protein (CREB)-binding protein cointegrator associate protein (p/CIP) [also designated amplified in breast cancer-1 (AIB1), receptor-associated coactivator 3 (RAC3), activator of thyroid and retinoic acid receptor (ACTR), thyroid hormone receptor activator molecule 1 (TRAM-1), or steroid receptor coactivator-3 (SRC-3)] (3–11). Members of this SRC-1 family have been shown to act as coactivators for steroid, retinoid, and thyroid hormone receptors, perhaps by modifying transcriptionally repressed chromatin or by enhancing stabilization of transcriptional preinitiation complexes (12–14). This coactivation function is mediated through direct ligand-dependent interaction with these receptors, which enhances the ligand-dependent transcription of target genes (15). Factors such as p300 and CREB-binding protein have been identified as part of the coactivator complex, functioning as a point of integration between ERα and other signaling pathways (16–18). The enhancement of the transcriptional activation of the steroid receptor superfamily by SRC-1 family members (19–21), as demonstrated in transfection experiments, suggests a potential role of SRC-1 family members in the development of normal estrogen target tissues and potentially in breast cancer formation. This is supported by the phenotype of SRC-1 null mice (22), which exhibit decreased growth and development of target organs (such as the uterus, prostate, testis, and mammary gland) in response to steroid hormones and by the finding of amplification of the SRC-1 family member AIB1 in some breast cancers.

Given the ability of SRC-1 to interact directly with steroid receptors and to enhance steroid receptor-dependent signaling, we expected that SRC-1 would be expressed in the same cells as the activator proteins such as ERα or progesterone receptor (PR) in normal hormone-responsive tissues. Although SRC-1 mRNA was detected in many tissues and cell lines (8, 23), the expression of SRC-1 protein at the cellular level has not previously been addressed. Experiments were designed to test the hypothesis that SRC-1 was expressed in the same cells as the ERα or PR. We chose the rat mammary gland as the model system because the development of the mammary gland is influenced by hormonal and growth factor signals, and this estrogen-responsive tissue has been a unique organ for the study of hormonal action, development, and tumorigenesis (24–26). The essential role of ERα and PR in mammary gland development has been confirmed by knockout mice lacking functional receptors. ERα knockout mice display grossly impaired ductal epithelial proliferation and branching (27, 28), and PR knockout mice display significant ductal development but decreased arborization and an absence of alveolar differentiation (29). To determine the precise localizations of ERα and SRC-1 within the mammary gland, we examined the

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Abbreviations: SRC-1, steroid receptor coactivator 1; ERα, estrogen receptor α; PR, progesterone receptor; AIB1, amplified in breast cancer-1; GST, glutathione *S*-transferase; FITC, fluorescein isothiocyanate.

‡To whom reprint requests should be addressed at: Department of Internal Medicine, Division of Hematology/Oncology, University of Virginia Health Sciences Center, Box 513, Charlottesville, VA 22908. e-mail: mj5x@virginia.edu.

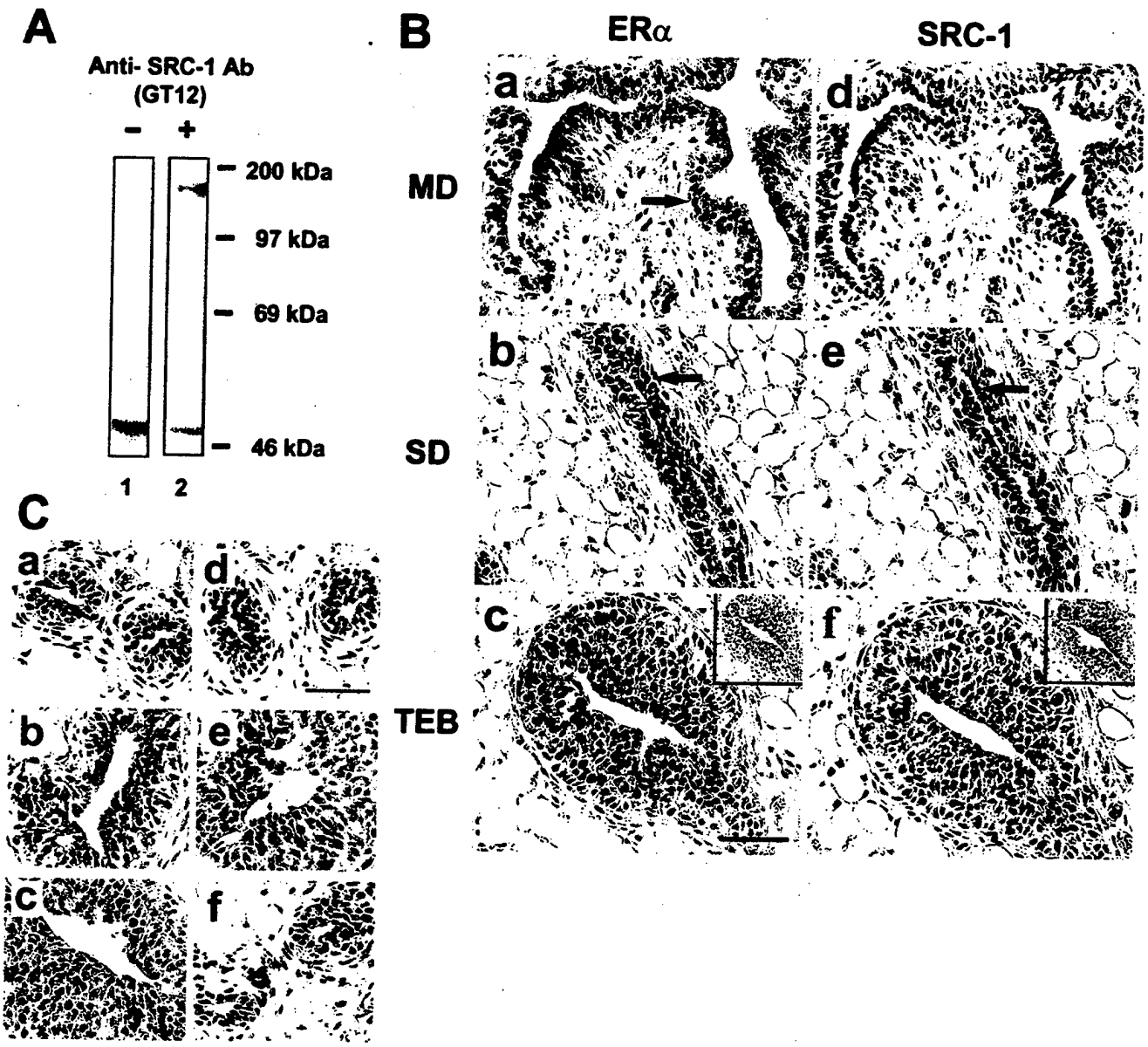

FIG. 1. Expression of SRC-1 and ERα in the rat mammary gland. (*A*) Mouse anti-SRC-1 (GT12) antibody recognized rat SRC-1 (lane 2) as demonstrated by Western blot analysis of GT12 using rat uterine tissue lysate. The endogenous rat IgG from tissues was detected by the secondary antibody, and this signal was also detected without primary antibody (lane 1). (*B*) Immunohistochemical staining of ERα (*a–c*) and SRC-1 (*d–f*) on adjacent sections from main ducts (MD), small ducts (SD), and terminal end buds (TEB) of the mammary gland from 3-week-old virgin female rats. Control specimens stained without primary antibodies are shown in *c* and *f* (*Insets*). The arrows are pointing to ERα-positive cells that are found in a layer closer to the basement membrane and SRC-1-positive cells that exist in a more luminal layer. (*C*) Immunoreactive SRC-1 was detected by both anti-SRC-1 antibodies, GT12 (*a*) and M-20 (*d*), in rat mammary gland. A control specimen (*c*) stained without primary antibody showed no staining signal. Preabsorption of GT12 or M-20 antibodies with SRC-1 fusion protein (*b*) or M-20-specific peptide (*e*), respectively, out-competed the staining signal. M-20 peptide did not diminish the staining signal detected by GT12 (*f*). Sections were counterstained with Harris hematoxylin. (Bars = 50 μm in *B* and *C*.)

expression of ERα and SRC-1 in the rat mammary gland by immunohistochemistry.

MATERIALS AND METHODS

Animals. Wistar–Furth and Sprague–Dawley female rats were purchased from Harlan Sprague–Dawley (Indianapolis) and treated according to National Institutes of Health and University of Virginia guidelines for the care and use of animals. An s.c. injection of 1 μg of estrogen benzoate or vehicle (sesame oil) was given to the rats. Later (24 h), the mammary gland and uterus were removed and processed for antibody staining or Western blot analysis.

Generation of Antibody. Human SRC-1 from amino acid 363 to the carboxyl terminus was fused to glutathione *S*-transferase (GST), and *Escherichia coli*-expressed GST–SRC-1 fusion protein was used to generate the monoclonal SRC-1 antibodies (GT12 and GT111). Tissue culture supernatants of GT12 and GT111 were used for both Western blot analysis and immunohistochemical staining.

Western Blot Analysis. Whole uterine tissue lysate was prepared by homogenization in RIPA buffer (50 mM Tris·HCl, pH 7.5/1 mM EDTA/150 mM NaCl/1% Triton X-100/1% deoxycholic acid/1 mM DTT/1 μg/ml leupeptin/1 μg/ml aprotinin/100 μg/ml PMSF). Protein of whole cell lysate (300 μg) was separated by electrophoresis on 7.5% polyacrylamide gels containing 1% SDS. Western blot analysis was carried out

according to procedures previously published (30) with GT12 at a 1:10 dilution.

Immunohistochemistry. An indirect immunoperoxidase method was used to identify ERα-, SRC-1-, or PR-positive cells. Tissues were fixed in cold 2% paraformaldehyde in PBS for 2 h, and 5-μm paraffin sections were heated in a microwave oven (900 watt, high power) in 10 mM citric buffer, pH 6.0, for antigen retrieval, treated with 0.3% H_2O_2 in methanol, and blocked with avidin D/biotin blocking solutions. Sections were then incubated with appropriate 10% normal serum, primary antibodies, appropriate secondary biotinylated antibodies, avidin-biotin complex, and diaminobenzidine substrates. ERα was detected with a rabbit anti-ERα IgG (MC-20; 1:400; Santa Cruz Biotechnology) or a mouse anti-ERα IgG (6F11; 1:50; NovoCastra, Newcastle, U.K.). SRC-1 was detected with mouse anti-SRC-1 antibodies (GT12; 1:2 or GT111; 1:1) or a goat anti-SRC-1 IgG (M-20; 1:50; Santa Cruz Biotechnology). PR was detected with a mouse anti-PR IgG (MA1–410; 1:25; Affinity BioReagents, Neshanic Station, NJ).

For SRC-1/ERα dual immunofluorescent labeling, sections were incubated sequentially with GT12 (1:2), biotinylated horse anti-mouse IgG (1:1000), Texas Red-conjugated streptavidin (1:100; Vector Laboratories), MC-20 (1:100), and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:200; Jackson ImmunoResearch). Alternatively, sections were incubated sequentially with 6F11 (1:50), biotinylated horse anti-mouse IgG (1:500), FITC-conjugated streptavidin (1:200; Vector Laboratories), M-20 (1:25), and Texas Red-conjugated donkey anti-goat IgG (1:100; Jackson ImmunoResearch). For PR/ERα dual labeling, sections were incubated sequentially with MA1–410 (1:25), biotinylated horse anti-mouse IgG (1:500), FITC-conjugated streptavidin (1:100), MC-20 (1:100), and Texas Red-conjugated goat anti-rabbit IgG (1:200; Jackson ImmunoResearch). For PR/SRC-1 dual labeling, sections were incubated sequentially with MA1–410, biotinylated horse anti-mouse IgG, FITC-conjugated streptavidin, M-20, and Texas Red-conjugated donkey anti-goat IgG. All procedures were done at room temperature. Slides were examined with a Zeiss Axioskop microscope equipped with appropriate fluorescence filter sets. Images were taken with a SenSys charge-coupled device camera (Photometrics, Tuscon, AZ) and IPLAB SPECTRUM software (Signal Analytics, Vienna, VA).

RESULTS

Expression of SRC-1 and ERα in Mammary Gland. Human SRC-1 from amino acid 363 to the carboxyl terminus was fused to GST, and the GST–SRC-1 fusion protein was used to generate the mouse monoclonal antibodies GT12 and GT111. The ability of the mouse monoclonal antibody GT12 to specifically recognize rat SRC-1 was confirmed by Western blot analysis in which a single 160-kDa band was detected (Fig.

Table 1. Distribution of ERα or SRC-1 immunoreactive cells in the epithelium of mammary gland in 3-week-old female rats

Region	ERα	SRC-1
Main duct	29.46 ± 3.46	41.98 ± 3.10
Small duct	42.37 ± 1.77	32.43 ± 2.09
End bud	40.29 ± 3.11	18.21 ± 1.26

Cells expressing immunoreactive ERα or SRC-1 were counted from various regions (end buds, small ducts, and main ducts) of the mammary glands from adjacent sections as indicated in Fig. 1*B*. Values are mean percentages of positive nuclei (±SD), with four animals in each group. For ERα immunoreactive cells, a total of 2,831 cells from 6 main ducts, 5,690 cells from 33 small ducts, and 7,448 cells from 31 end buds was counted. For SRC-1 immunoreactive cells, a total of 2,657 cells from 6 main ducts, 5,437 cells from 33 small ducts, and 6,954 cells from 31 end buds was counted.

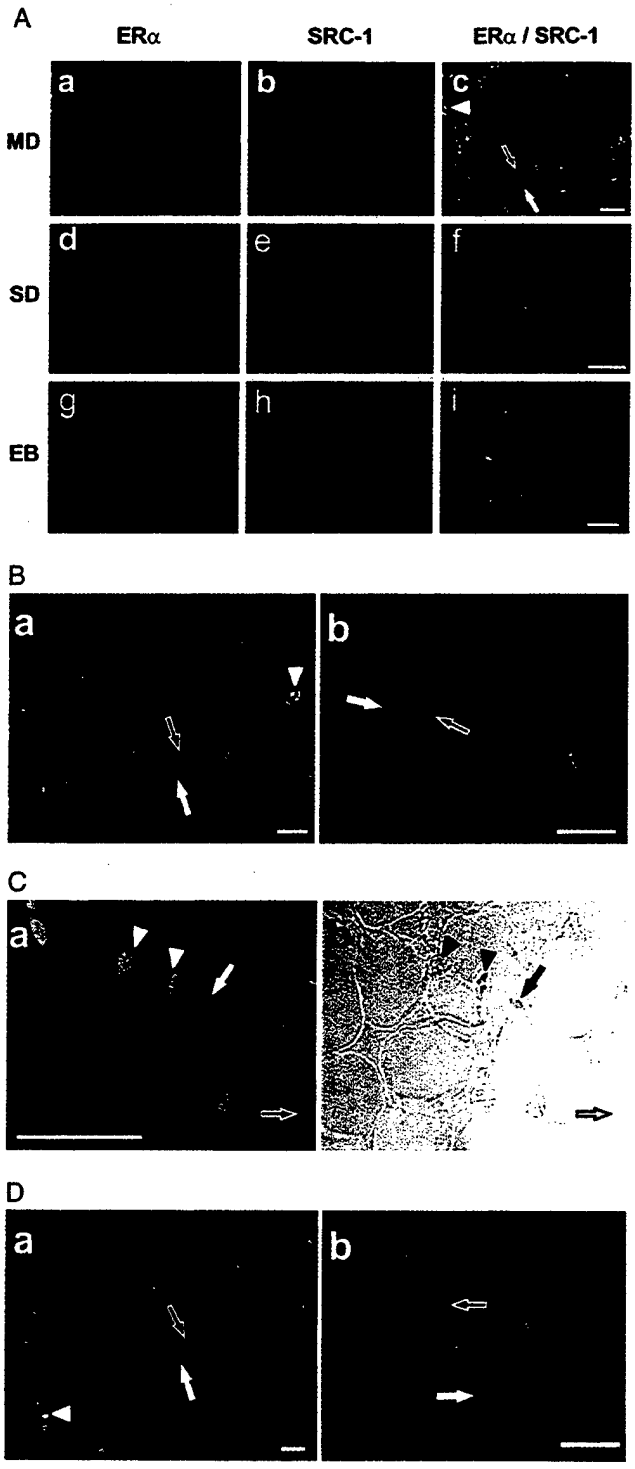

FIG. 2. Segregation of SRC-1 expression from ERα-positive cells as illustrated by the dual immunofluorescent labeling of SRC-1 and ERα. (*A*) Mammary glands from 3-week-old virgin female rats were stained simultaneously for SRC-1 (*b*, *e*, and *h*; red) with GT12 and for ERα (*a*, *d*, and *g*; green) with MC-20. Green and red images were superimposed (*c*, *f*, and *i*). Main duct (MD), small duct (SD), and end bud (EB) are shown. (*B*) The discrete distribution pattern of ERα (green) and SRC-1 (red) was confirmed with the combination of 6F11 and M-20 antibodies (*a* and *b*). (*C*) Stroma expressing ERα alone (green), SRC-1 alone (red), or both (yellow) were detected with GT12 and MC-20 antibodies (*a*) as in *A*. The phase contrast image from *a* is shown in *b*. (*D*) Staining from 10-week-old virgin female rat mammary gland also demonstrated the segregation of SRC-1 from ERα in epithelial cells. MC-20 and GT12 were used as the primary antibodies. Solid arrow, Cells expressing only ERα; open arrow, cells expressing only SRC-1; solid arrowhead, cells expressing both ERα and SRC-1. (Bar = 100 μm.)

1*A*). Immunohistochemical staining performed with GT12 and rabbit anti-ERα (MC-20) antibodies on adjacent sections of the rat mammary gland demonstrated that both SRC-1 and ERα antibodies stained subpopulations of mammary epithelial cells in various regions of the gland (Fig. 1*B*, arrows). The ERα-positive cells were found in a layer closer to the basement membrane, and the SRC-1-positive cells existed in a more luminal layer.

Control specimens stained with antibodies previously preabsorbed by the GST–SRC-1 fusion protein (Fig. 1*Cb*) or the ERα-specific MC-20 antigen (data not shown) yielded only background staining. Preabsorption of the GT12 antibody with GST protein alone did not diminish the specific immunostaining signal (data not shown). To ensure that the immunostaining profile was valid, we tested a second SRC-1 monoclonal antibody, GT111, and observed the same staining pattern (data not shown). In addition, we tested a goat anti-SRC-1 IgG (M-20; epitope corresponding to mouse amino acids 1386–1405) that recognizes a different portion of SRC-1 than GT12 or GT111 and again saw the same pattern of staining (Fig. 1*Cd*). As anticipated, the immunoreactivity could be eliminated by preabsorption of this antibody with the specific SRC-1 peptide antigen (Fig. 1*Ce*). The SRC-1 M-20-specific peptide did not compete with the stained signal detected by GT12 (Fig. 1*Cf*), confirming that M-20 and GT12 indeed recognize different epitopes of the rat SRC-1 protein.

We and others previously found that ERα was expressed in only a subset of cells in mammary epithelium (Fig. 1 *Ba–Bc*, arrows) (31, 32). In this study, we found that SRC-1 was also expressed in only a subset of mammary epithelial cells, with distinct patterns of expression when compared with ERα expression (Fig. 1 *Bd–Bf*, arrows). The percentage of mammary epithelial cells from 3-week-old female rats expressing ERα or SRC-1 in the main ducts, small ducts, and end buds is quantitated in Table 1. We found that ERα was most highly expressed in the small ducts and end buds, and SRC-1 was expressed in a complementary fashion, with the main duct epithelium having the highest percentage of SRC-1 positive cells. These initial results suggested that ERα and SRC-1 might have distinct patterns of expression within the mammary epithelium.

Discrete Pattern of SRC-1 and ERα Distribution in Mammary Gland. To test whether ERα and SRC-1 are expressed in the same or different mammary epithelial cells, we undertook dual immunofluorescent labeling studies with a combination of rabbit anti-ERα (MC-20) and mouse anti-SRC-1 (GT12) antibodies. The immunostaining signals were detected by FITC-conjugated anti-rabbit IgG in green for ERα (Fig. 2 *Aa*, *Ad*, and *Ag*) and biotinylated anti-mouse IgG and Texas Red-conjugated streptavidin in red for SRC-1 (Fig. 2 *Ab*, *Ae*, and *Ah*). Surprisingly, we found that cells expressing SRC-1 are nearly completely segregated in the mammary epithelium from cells expressing ERα in all regions of the mammary gland examined, including the main ducts (Fig. 2 *Aa–Ac*), small ducts (Fig. 2 *Ad–Af*), and end buds (Fig. 2 *Ag–Ai*). Merging the two images (Fig. 2 *Ac*, *Af*, and *Ai*) revealed only very rare cells coexpressing ERα and SRC-1 as would be indicated by a yellow signal. When these results were quantitated, less than 2% of the mammary epithelial cells in ducts or end buds expressed both ERα and SRC-1 (Table 2). These immunostaining results were confirmed with a second set of antibodies that recognize different epitopes of SRC-1 and ERα (Fig. 2*B*). In these fluorescent images from immature rats, it appears as though ERα and SRC-1 define two distinct layers in the ductal epithelium. This is consistent with our initial observation (Fig. 1*B*). The ERα-positive cells are found in a layer closer to the basement membrane, whereas the SRC-1-positive cells exist in a more luminal layer, suggesting that SRC-1 and ERα are markers of distinct subpopulations of the mammary epithelium.

We next examined whether the expression of ERα and SRC-1 was mutually exclusive in all cell types within the mammary gland. In contrast to the mammary epithelium, a substantial number of cells within the mammary stroma expressed both SRC-1 and ERα (Fig. 2*C*), implying a potential different function for SRC-1 in the mammary epithelium compared with mammary stroma. In addition, we examined whether the segregation of ERα and SRC-1 expression to distinct cells in the mammary epithelium was unique to early postnatal mammary development in the rat or was preserved in adult rats. We found that, as was the case in immature rats, ERα and SRC-1 were not coexpressed in the same cells in the mammary epithelium of mature virgin female rats (Fig. 2*D*). This finding indicates that the segregation of expression of ERα and SRC-1 was not altered during the maturation of the mammary gland under the control of ovarian steroids.

Estrogen-Stimulated PR Induction Does Not Require SRC-1. The discovery of a subpopulation of mammary epithelial cells expressing ERα but lacking SRC-1 allowed us to ask whether SRC-1 expression was required for one of the critical ERα functions in the epithelium of the mammary gland, namely, the induction of PR. The PR gene has been shown to be a direct target of ERα regulation, is detected only in a subset of epithelial cells of the mouse mammary gland, and has been colocalized to ERα-expressing cells in normal human breast epithelium (33–35). Immature female rats were treated with vehicle alone (Fig. 3 *Aa–Ac*) or with estrogen benzoate (Fig. 3 *Ad–Af*) and the expression of SRC-1, ERα, and PR was examined in the mammary gland. We found that, as with mature rats, exposure of the mammary epithelium to estrogen did not alter the segregated pattern of expression of SRC-1 and ERα (Fig. 3 *Aa* and *Ad*). In vehicle-treated animals only a minority of mammary epithelial cells expressed PR, and these were also uniformly ERα-expressing cells (Fig. 3*Ab*). Significantly, treatment of animals with estrogen led to a striking increase in the proportion of cells in which PR was detectable, and again these were exclusively the ERα-positive cells (Fig. 3*Ae*). Consistent with the coexpression of ERα and PR and the segregated pattern of expression of ERα and SRC-1, PR and SRC-1 expression was also mutually exclusive (Fig. 3 *Ac* and *Af*) in the mammary epithelium. No PR was detected in the stromal compartment of the rat mammary gland and estrogen treatment did not induce PR expression in these stromal cells expressing both ERα and SRC-1 (Fig. 3 *B* and *C*; data not

Table 2. ERα and SRC-1 immunoreactive cells on dual fluorescent labeling in the epithelium of mammary gland in 3-week-old female rats

Region	ERα-positive/ SRC-1-negative	ERα-negative/ SRC-1-positive	ERα-positive/ SRC-1-positive	ERα-negative/ SRC-1-negative
Main duct	26.62 ± 5.85	37.12 ± 4.05	1.44 ± 0.67	34.82 ± 9.22
Small duct	34.82 ± 2.81	33.17 ± 4.04	1.64 ± 0.59	28.13 ± 4.28
End bud	34.60 ± 2.49	17.31 ± 2.44	1.31 ± 0.25	45.84 ± 2.54

Cells expressing immunoreactive ERα, SRC-1, or both were counted from various regions (end buds, small ducts, and main ducts) of the mammary glands as indicated in Fig. 2*A*. Values are mean percentages of positive nuclei (±SD), with four animals in each group. A total of 2,183 cells from 4 main ducts, 3,911 cells from 19 small ducts, and 4,274 cells from 18 end buds was counted.

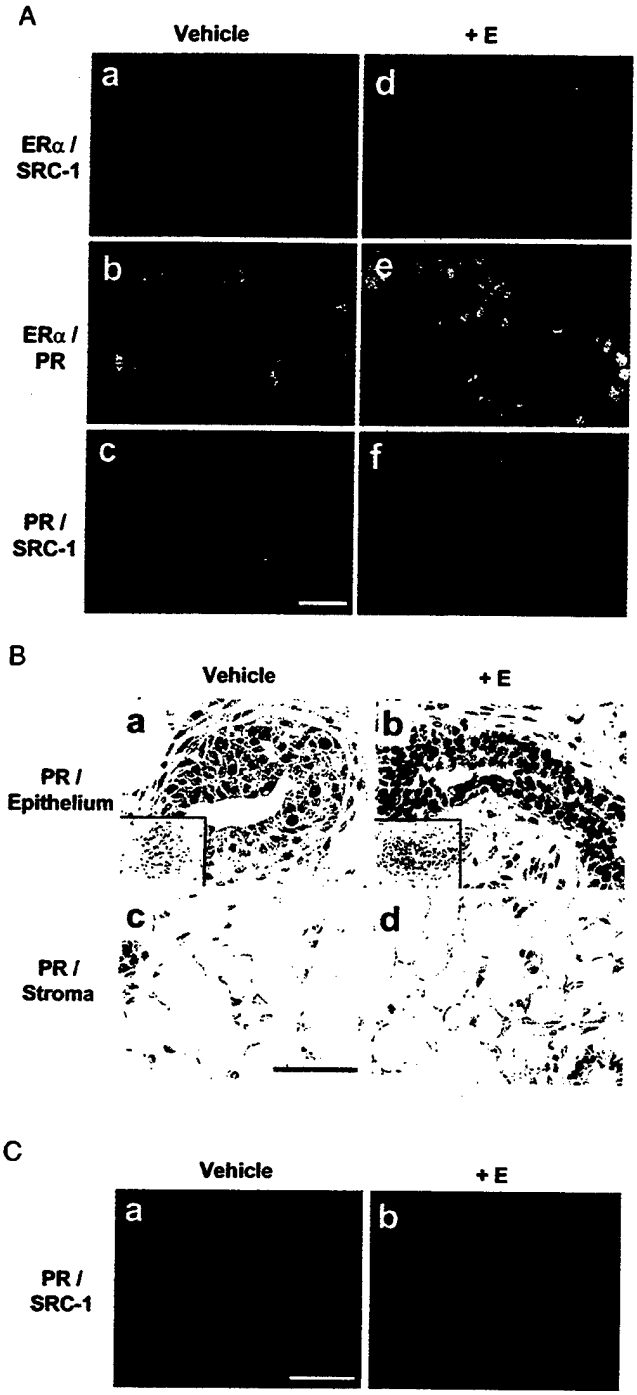

FIG. 3. Segregation of SRC-1 from both ERα and PR in rat mammary epithelial cells when treated with estrogen. Mammary gland from 3-week-old virgin female rats treated with vehicle (sesame oil) or 1 μg of estrogen benzoate (+E) for 24 h were used. (*A*) Dual immunofluorescent labeling of ERα/SRC-1, ERα/PR, and PR/SRC-1. (*a* and *d*) ERα (green) and SRC-1 (red) were stained simultaneously with MC-20 and GT12. (*b* and *e*) ERα (red) and PR (green) were stained simultaneously with MC-20 and anti-PR IgG (MA1–410). (*c* and *f*) PR (green) and SRC-1 (red) were stained simultaneously with MA1–410 and M-20. (Bar = 100 μm.) (*B*) Expression of PR in both epithelium and stroma of the mammary glands. Control specimens stained without primary antibody are shown in insets. (Bar = 50 μm.) (*C*) Dual immunofluorescent labeling of PR and SRC-1 in stroma of the mammary glands. The staining was performed as in *A*. (Bar = 100 μm.)

shown), suggesting that coexpression of ERα and SRC-1 was neither necessary nor sufficient to induce PR expression.

DISCUSSION

SRC-1 has been identified as a coactivator for ERα based on its ability to interact directly with agonist-bound ERα and to potentiate ERα-dependent signaling in transient transfection experiments. The coactivation function of SRC-1 has also been documented for PR. Hence, we expected that SRC-1 would be expressed in the same cells in which ERα or PR was present in normal hormone-responsive tissues. In this study, we found unexpectedly that expression of SRC-1 and ERα is segregated in the rat mammary epithelium, an estrogen-responsive tissue. In addition, we have demonstrated that induction of PR expression in mammary epithelium does not require coexpression of SRC-1 and that the presence of SRC-1 in stromal cells coexpressing ERα was not sufficient to facilitate PR induction by estrogen. Finally, the pattern of expression of ERα in cells closer to the basement membrane and the contrasting expression of SRC-1 in cells that are more luminal implies the existence of at least two distinct cell subpopulations in the rat mammary ductal epithelium.

The recent description of SRC-1 null mice suggests that SRC-1 is required for efficient proliferation and differentiation of the mammary gland in response to estrogen and progesterone, because the mammary glands of these mice showed less alveolar development during pregnancy and responded to estrogen and progesterone treatment with only partial ductal growth (22). Our results here, showing that SRC-1 and ERα (or PR) are expressed in distinct subpopulations of mammary epithelial cells and that the coexpression of SRC-1 and ERα does not facilitate the PR gene expression in mammary stromal cells, are in concert with the mammary gland phenotype observed in the SRC-1 null mice. Because SRC-1 and ERα/PR are expressed in distinct cells within the mammary epithelium, SRC-1 is apparently not directly involved in estrogen- or progesterone-initiated signaling in the mammary epithelium. This strongly indicates that, conversely, the role of SRC-1 in morphogenesis of the epithelium is likely to interact with other signaling molecules whose identities remain to be defined. To the extent that morphogenesis is affected in the SRC-1 null mice, it remains unclear whether this is caused by a malfunctioning of the SRC-1-positive epithelial cells, the SRC-1-positive stromal cells, or both. Paracrine effects including epithelial–stromal and epithelial–epithelial interactions have been implicated in the action of ovarian steroids in the morphogenesis of the normal breast (33–39) and could play a role in the mammary gland phenotype observed in SRC-1 null mice.

The demonstration that the segregation of expression of ERα and SRC-1 was not altered during the maturation of the mammary gland under the control of ovarian steroids indicates that ovarian steroids are not able to increase the expression of SRC-1 in ERα-positive cells to potentiate the estrogen signaling. In the immature gland, two distinct layers of epithelial cells were clearly visualized in small ductal areas (Fig. 2*B*). Maturation of the mammary gland after puberty results in a less clear distinction of the layers of epithelial cells in small ductal areas (Fig. 2*D*). Interestingly, SRC-1-expressing cells are still adjacent to the ERα-expressing cells in adult rats. This suggests that the organization of the mammary epithelium may be important in mediating its complex paracrine response to estrogen. In addition, SRC-1 and ERα can serve as cellular markers of distinct subpopulations of the mammary epithelium, with unknown function.

Our observations that PR was not induced in ERα- and SRC-1-coexpressing stromal cells or in SRC-1-expressing epithelial cells during estrogen treatment support the hypothesis that SRC-1-coactivating function for ERα is neither necessary nor sufficient for PR expression in normal mammary gland. It is possible that other SRC-1 family members such as glucocorticoid receptor-interacting protein 1/transcriptional interme-

diary factor 2 or p300/CREB-binding protein cointegrator associate protein/AIB1 are involved in ERα coactivation in cells that do not express SRC-1, as suggested by other investigators (22). Examination of the expression of other SRC-1 family members in ERα-positive cells will be necessary to test this hypothesis. *In vitro* studies suggest that SRC-1 could partner with various nuclear receptors for its coactivation function. Based on our observation that SRC-1 is segregated from ERα and PR in mammary epithelium, it is interesting to speculate that SRC-1 may have cell- or tissue-specific partners for its coactivation function. The second form of ER, ERβ (40), or other nuclear receptors could be the partner for SRC-1 coactivation function in the mammary gland. On the other hand, we have found that the segregation of SRC-1 from ERα is tissue-specific and may serve to expand the spectrum of hormonal response in various estrogen target organs. This view is supported by our observation that in uterine epithelium SRC-1 and ERα are colocalized in the vast majority of uterine epithelial cells (M.-H.J., unpublished data).

One possible limitation of our findings, which are based on immunohistochemical staining, is that cells expressing extremely low levels of SRC-1 or ERα might not be detectable by this technique. We cannot rule out the possibility that levels of SRC-1 undetectable by immunohistochemical means may be sufficient for coactivation in cells expressing a high level of ERα. However, estrogen-stimulated PR induction occurred only in mammary epithelial cells expressing ERα but not in adjacent cells where the SRC-1 was easily detectable. Rather than playing the role of an ERα or PR coactivator in cells in which it cannot be detected, it is more likely that SRC-1 is in fact playing some cell type-specific role in the mammary epithelial cells, which do express SRC-1 to high levels. This view is further supported by the observation that PR was not induced in stromal cells that coexpressed both ERα and SRC-1. Our data strongly suggest a potential cell type-specific role of SRC-1 that does not involve direct interaction of SRC-1 with ERα or PR.

Finally, although ERα and SRC-1 are not coexpressed in the normal mammary epithelium, it is interesting to speculate, given the finding of amplification of the SRC-1- related gene AIB1 in some breast cancers (7), that the ectopic coexpression of ERα and an SRC-1 family coactivator may play a role in the growth stimulatory properties of estrogen in breast cancer.

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Reconstitution of Estrogen-Dependent Transcriptional Activation of an Adenoviral Target Gene in Select Regions of the Rat Mammary Gland*

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ABSTRACT

Estrogen regulates proliferation and morphogenesis of mammary ductal epithelium by interacting with a specific intracellular estrogen receptor (ER) that acts as a hormone-dependent transcriptional regulator of gene expression. The mechanisms by which ER regulates transcription in response to estrogen have been analyzed extensively in tissue culture and in cell-free systems. These studies have demonstrated that the transcriptional activity of ER is strongly influenced by cellular context and highlight the need to address ER transcriptional activity in an appropriate cellular background. Thus, to gain insight into the mechanistic role of ER in mammary epithelial morphogenesis, we have used an adenoviral gene delivery strategy to introduce an estrogen-responsive reporter gene into the mammary epithelium and to monitor the activity of endogenous ERs in their natural environment where cellular context including stromal-epithelial interactions can be taken into account. Using this approach, we first demonstrated highly efficient adenoviral delivery throughout the mammary epithelium using a β-galactosidase (βgal) reporter gene under the control of the constitutively active cytomegalovirus (CMV) promoter. Next, we constructed an adenoviral vector by substituting the CMV promoter with an estrogen-dependent promoter fragment-linked βgal (Ad-ERE-tk-βgal). This adenoviral reporter system provides evidence that ER positive mammary epithelial cells display a differential sensitivity in a region-specific manner toward estrogen induction. Our data suggest that the availability of factor(s) other than ER is necessary for ER-mediated gene activation and may be important in modulating the differential responses of mammary epithelial cells to estrogen. (*Endocrinology* **139:** 2916–2925, 1998)

THE DEVELOPMENT of the mammary gland occurs primarily postnatally and is directed by a complex interplay between hormonal (polypeptide and steroid) and growth factor signals (1, 2). Progesterone and estrogen are the principle steroid hormones involved in normal breast development and tumorigenesis (3–7). During pregnancy, progesterone and estrogen promote growth and differentiation of normal mammary tissue by regulating ductal proliferation and branching, alveolar formation (8), and lobulo-alveolar development (9). In the case of mammary gland tumorigenesis, the effects of progesterone and estrogen can be either stimulatory or inhibitory or both, and such effects are dose and stage dependent (10, 11). The hormonal effects are known to be mediated by specific high affinity intracellular receptor proteins that are members of a superfamily of related transcription factors (12–16). Studies on the ontogeny of mouse mammary gland responsiveness to ovarian steroid hormones have indicated that receptors for estrogen and progesterone (ER and PR, respectively) are present in both stromal and epithelial cells. The estrogen and progesterone receptors in epithelial cells are responsive to their ligands at 4 and 7 weeks of age, respectively (17, 18). The essential role of these receptors in mediating mammary developmental responses to estrogen and progesterone has been confirmed recently by the generation of null mutant mice lacking functional receptors for both hormones (19–21). These mice display grossly impaired ductal epithelial proliferation and branching in the case of the estrogen receptor null mutants and significant ductal development but decreased arborization and an absence of alveolar differentiation in the case of the progesterone receptor null mutants.

The mechanism by which steroid hormone receptors mediate hormone-induced signal transduction has been studied extensively in tissue culture and cell-free systems. Binding of steroids to their cognate receptors results in the formation of activated receptor dimers that bind to specific enhancer DNA elements located in the promoter regions of hormone-responsive genes (22, 23). Ligand-dependent activation is accompanied by a removal of receptor-bound corepressor proteins (24) that inhibit transcriptional activation by steroid receptors and an induction of binding of coactivator proteins that facilitate functional interaction of steroid receptors with the general transcription machinery (15, 24, 25, 26). The activation or repression of specific genes by steroid receptors represents the manifestation of the hormonal response. Reconstitution of steroid receptor-dependent transcriptional responses in cultured cells has demonstrated that the receptors can be activated not only by their cognate ligand but also by intracellular signaling pathways

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initiated by growth factors and other extracellular signals in a ligand-independent manner (27). However, the impact of these ligand-independent pathways on ER or PR mediated regulation of transcription *in situ* in the mammary gland has not been established. Further, little information is available to date on the factors that influence localized ER and PR mediated regulation of transcription during mammary tissue development or tumorigenesis.

The objective of the present study was to develop a strategy that ultimately would allow us to localize and monitor changes in ER-dependent transcriptional responses *in vivo* in the mammary gland that occur as a function of developmental status in the presence or absence of hormonal or growth factor stimuli or in response to chemical or hormonal carcinogens. Our approach was to use an adenoviral gene delivery system (28–30) to introduce an exogenous estrogen-responsive reporter construct into the rat mammary gland. Using this system, we reconstituted estrogen-dependent reporter gene expression *in situ* and localized this response to a subpopulation of epithelial cells located in the branched small ducts. A significant portion of the ductal epithelium appears to be refractory to estrogen despite the presence of high concentrations of ERs. These data suggest that estrogen sensitivity of the ductal epithelium is regulated locally by the availability of additional factors other than ER that are necessary to impart a transcriptional regulation of ER target genes in mammary epithelium.

Materials and Methods

Tissue culture cells and experimental animals

Hela, CV-1, 3T3, MCF-7, and 293 cells (American Type Culture Collection, Rockville, MD) were maintained in DMEM containing 10% FBS. Tryptose phosphate (0.26 g/liter) was added during homologous recombination and plaque assays. Medium components were obtained from Gibco BRL (Grand Island, NY).

Female Wistar Furth rats (28 days old) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). They were anesthetized and either sham-operated or ovariectomized before experimentation. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Recombinant adenovirus construction and large-scale production

Replication defective recombinant adenoviruses expressing β-galactosidase (β-gal) under the control of the *cis*-acting estrogen response element (ERE) and either the E1b or thymidine kinase (tk) minimal promoters were constructed. For pERE-tk-βgal shuttle vector construction, the 191 bp *XbaI-BglII* ERE-tk fragment containing a single copy of the ERE sequence upstream of the tk promoter was isolated from plasmid pERE15 and ligated upstream of a 3.4-kb *HindIII-DraI* β-galactosidase fragment from pCH110 (Pharmacia Biotech Inc., Piscataway, NJ), and the 153-bp poly A^+ fragment from SV40 DNA in the pXCJL Ad vector (31). For the pERE4-Elb-βgal shuttle vector construction, a synthetic oligonucleotide containing four copies of the ERE sequence located upstream of the E1b minimal promoter (32) was subcloned into the pqE1sp1 adenoviral shuttle vector (33). PXCJL, pqE1sp1, and pJM107 (containing the adenoviral genome) were obtained from Dr. Frank Graham (McMaster University, Hamilton, Ontario, Canada). Both adenoviral shuttle vectors were $CsCl_2$-purified and were then cotransfected with pJM107 into 293 cells using *N*-(1-(2, 3-dioleoyloxyl)propyl)-*N,N,N*-trimethylammoniummethyl sulfate mediated transfection method according to the manufacturer's instructions to allow homologous recombination to occur (Boehringer Mannheim Biochemicals, Indianapolis, IN). Individual plaques were isolated and amplified in 293 cells. Viral DNAs were prepared, and the recombinant adenovirus (Ad) was identified by PCR and Southern analyses according to the method of Graham and Prevec (34). Selected clones of Ad-ERE-tk-βgal and Ad-ERE4-Elb-βgal were obtained by plaque purification and propagated in 293 cells (34). Cells were harvested 36–48 h after infection. Cell pellets were then resuspended in PBS (50 mM Na phosphate, 100 mM NaCl, pH 7.4; PBS), lysed by three freeze/thaw cycles, centrifuged at 1000 × *g* for 5 min to remove cell debris, and the virus was purified by $CsCl_2$ gradient centrifugation. Concentrated virus was immediately dialyzed, aliquoted, and stored at −80 C. Viral titers were determined by OD 260 nm measurement or plaque assay. The control virus Ad-CMV-βgal where βgal is under the control of the constitutive cytomegalovirus (CMV) promoter used in this study was constructed in a similar manner.

Assessment of in vitro estrogen-induced transactivation of ERE-reporter activity in cultured mammary epithelial cells via infection with an adenoviral vector

To evaluate the effects of estrogen and antiestrogen on the expression of recombinant adenovirus reporter constructs in tissue culture cells, 4×10^5 MCF-7 cells were plated in six-well plates and deprived of estrogen for 1–3 days before transfection in phenol red-free DMEM containing 5% dextran-coated charcoal-stripped FBS (DCC-FBS) (35, 36). Transfection was done by exposing the culture cells to recombinant adenovirus for 2 h in serum-free and phenol red-free DMEM. Medium was then removed and replaced with fresh phenol red-free DMEM containing 5% DCC-FBS. 17-β estradiol (E_2, 10^{-8}–10^{-12} M) and/or ICI 164, 384 (10^{-7} M) were dissolved in ethanol and added to the medium for 24 h to demonstrate steroid specificity in transactivation of ER target genes. Cells were then fixed for X-gal staining or harvested for liquid β-galactosidase assay. Data are presented as the average of duplicate values. The experiments were repeated at least three times. E_2 was purchased from Sigma Chemical Co. (St. Louis, MO). ICI 164, 384 was obtained from Zeneca Pharmaceuticals (Macclesfield, UK).

Assessment of in vivo estrogen-induced transactivation of ERE-reporter activity in rat mammary gland via adenoviral vector infection

Twenty-eight-day-old female Wistar Furth rats were anesthetized and ovariectomized to reduce the circulating estrogen and progesterone. Ten days later, rats receiving the adenovirus were first anesthetized and infused with 10 μl adenovirus in conjunction with a vital tracking dye (indigo carmine, 50 μg/10 μl) through intraductal injection with a blunt-ended 20–26 gauge needle (37). At the same time, rats receiving hormonal treatment were given estrogen benzoate (EB) suspension in sesame oil (100 μg/0.2 ml) sc. Twenty-four hours later, the animals were then anesthetized, and the mammary fat pad was removed for X-gal staining. Rats were then euthanized with CO_2.

β-galactosidase assay and X-gal staining in cultured cells and in tissues

For β-galactosidase liquid assay in tissue culture cells, cell monolayers were rinsed once with PBS followed by the addition of 1 ml of 40 mM Tris HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl) and incubated on ice for 5 min. Cells were harvested, pelleted, and suspended in 100 μl ice-cold 0.25 M Tris HCl, pH 7.5. Cell extracts were prepared by three freeze/thaw cycles. Cytosols containing equal amount of protein were used for β-galactosidase activity assay (38).

For X-gal staining *in situ* in tissue culture cells, cells were rinsed twice with PBS and fixed with 0.5% glutaraldehyde in PBS for 5 min at room temperature. Cells were then rinsed twice with PBS and stained with X-gal staining solution (1.3 mM MgCl2, 15 mM NaCl, 44 mM HEPES, pH 7.4, 3 mM $K_3Fe(CN_6)$ 3 mM $K_4Fe(CN_6)$, and 0.5 mg/ml X-gal).

For X-gal staining in the mammary gland, rats were anesthetized and fat pads containing the mammary gland were removed. The staining procedure was performed according to the method described previously (39) with modification. The fat pads were fixed in fresh cold 2% paraformaldehyde solution containing 0.1 M PIPES, pH 6.9, 2 mM $MgCl_2$, 1.25 mM EGTA for 1–2 h, washed with PBS containing 2 mM $MgCl_2$ three

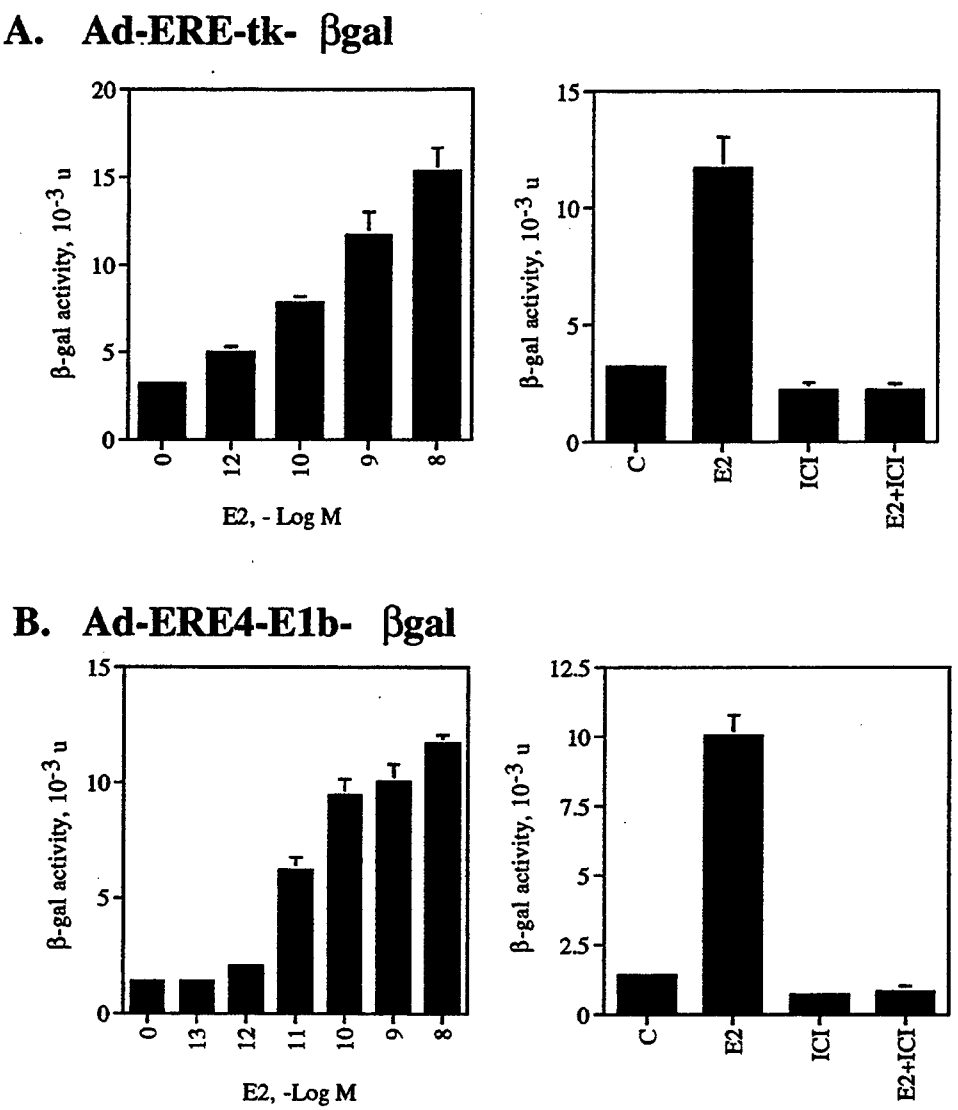

FIG. 1. Induction of β-galactosidase activity by E_2 treatment after Ad-ERE-tk-βgal or Ad-ERE4-E1b-βgal infection in MCF-7 cells. MCF-7 cells were deprived of estrogen and infected with Ad-ERE-tk-βgal (A) or Ad-ERE4-E1b-βgal (B) for 2 h in serum free DMEM. Media were removed and fresh media containing 5% DCC-FBS, and the indicated amount of E_2 were added to cells for an additional 24 h. ICI 164, 384 at a concentration of 10^{-6} M was used to block the effect of E_2 at the concentration of 10^{-9} M. Cell pellets were then collected and cytosols prepared. An equal amount of protein was used for β-galactosidase assay. Each *bar* represents the mean value from duplicates, the experiments were repeated at least three times.

times, and permeabilized with 0.02% NP40, 0.01% Na deoxycholate, and 2 mM $MgCl_2$ in PBS for 1 h. The fat pads were then stained immediately with staining solution containing 25 mM $K_3Fe(CN_6)$, 25 mM $K_4Fe(CN_6)$, 2 mM $MgCl_2$, 0.02% NP40, 0.01% Na deoxycholate, 0.5 mg/ml X-gal in PBS, pH 8.1 at 37 C for 12–16 h. After staining and photography, the glands were subsequently dehydrated, embedded in paraffin, and sectioned serially for microscope examination and photography.

Immunohistochemical analysis

The right and left no. 4 abdominal mammary glands from EB treated rats were sectioned into proximal and distal regions relative to the nipple and fixed in 4% paraformaldehyde. Tissues were embedded in paraffin and sectioned into 5-μm thick sections. The sections were then deparaffinized, rehydrated through graded alcohols followed by incubation in PBS. They were then incubated for 30 min each in 0.2% glycine and 0.3% hydrogen peroxide in methanol. Sections were rehydrated in PBS and blocked with 10% goat serum in PBS for 30 min followed by incubation overnight with rabbit anti-ER IgG, MC-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:200 dilution. Sections were incubated without primary antibody or with rabbit serum as control. Sections were rinsed several times in PBS (5 min each) and then incubated with biotinylated goat antirabbit secondary antibody at a 1:500 dilution for 15 min at 40 C, washed several times in PBS and then developed using the Vectastain ABC kit (Vector Labs., Burlingame, CA). All sections were counterstained with hematoxylin. Brown staining diaminobenzidine positive cells were visualized using a Zeiss Axioskop microscope (Carl Zeiss Inc., Thornwood, NY) at 40× magnification coupled to a Hamamatsu C5810 CCD camera (Hamamatsu Corp., Bridgewater, NJ) and were processed using Adobe Photoshop 4.0 (Adobe Systems, Inc., San Jose, CA).

Results

Estrogen-dependent activation of adenoviral reporter genes in MCF-7 breast cancer cells

To examine the feasibility of reconstituting estrogen-dependent reporter gene expression *in vivo* in the rat mammary gland, we constructed two recombinant adenovirus vectors bearing ERE-driven reporter constructs. The reporter constructs (Ad-ERE-tk-βgal and Ad-ERE4-E1b-βgal) contained one copy of the ERE located upstream of the thymidine kinase (tk) or four copies of the ERE located upstream of the adenoviral E1b minimal promoters (32) and the β-galactosidase reporter gene, respectively. To test hormone responsiveness of these reporter constructs in cultured cells, MCF-7 cells were infected first by these adenoviral expression vectors before exposure to estrogen. Twenty-four hours later,

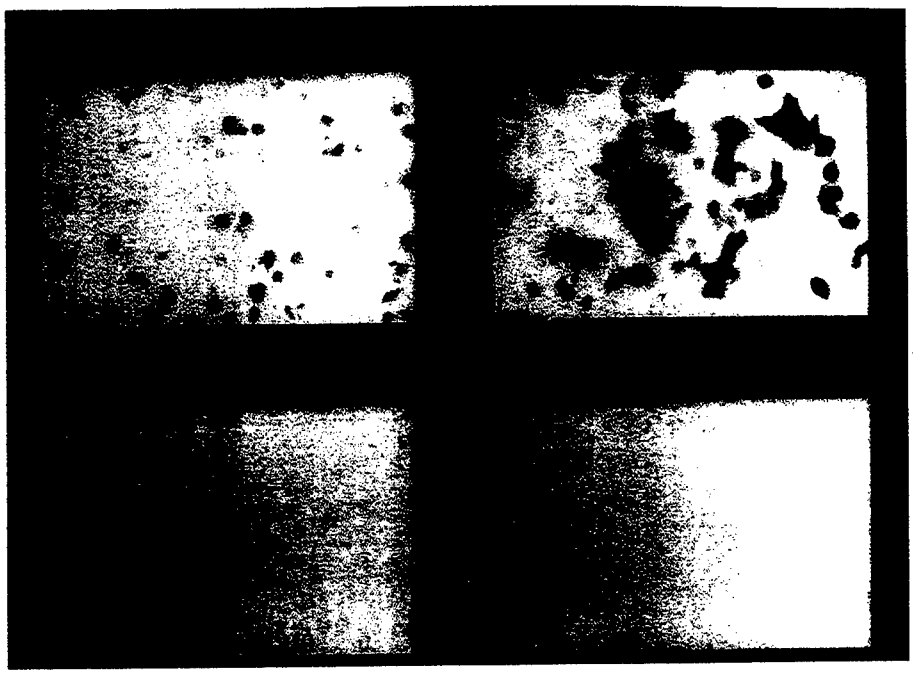

FIG. 2. X-gal staining of MCF-7 cells after E_2 and ICI 164, 384 treatments. MCF-7 cells were deprived of estrogen and infected with Ad-ERE4-E1b-βgal for 2 h in serum-free DMEM. Media were removed and fresh media containing 0.2% ETOH vehicle (A), 10^{-10} M E_2 (B), 10^{-7} M ICI 164, 384 (C), and 10^{-10} M E_2 and 10^{-7} M ICI 164, 384 (D) was added for an additional 24 h. Cells were then fixed with glutaraldehyde and stained with X-gal solution to visualize the *blue* cells in which the β-galactosidase was expressed.

FIG. 3. Intraductal injection of vital tracking dye into rat mammary gland. Four-week-old female Wistar-Furth rats were anesthetized and cannulated with a 22-gauge blunt-ended needle into the mammary main duct and 10 μl of the indigo carmine *blue* tracking dye (5 mg/ml in PBS) was infused within seconds into the mammary gland (A). The dye outlined the arborized structures consisting of primary, secondary, and tertiary branches and reached the end buds (B) (used with permission of Plenum Press; Ref. 40).

cell cytosols were prepared and analyzed for β-galactosidase activity. Figure 1 (A and B) shows that β-galactosidase expression is induced at comparable levels in MCF-7 cells infected with either Ad-ERE-tk-βgal or Ad-ERE4-E1b-βgal. Estrogen induces the expression of reporter activity in a dose-dependent manner, and the induction by estrogen is blocked in both cases by the presence of an estrogen receptor antagonist, ICI 162, 384.

The degree of cell infection obtained using the Ad-ERE4-E1b-βgal target virus is shown in Fig. 2. Panel A indicates that, in the absence of estrogen, approximately 30% of cultured cells express basal levels of β-galactosidase. However, incubation with estrogen resulted in robust expression of β-galactosidase activity in approximately 70% of the cells (panel B). Further, both the basal and estrogen induced activity were blocked by incubation with ICI 162, 384 (panels C and D). Thus, the adenoviral strategy for introducing synthetic target genes into cultured cells results in a high degree of infection, and the target gene retained responsiveness to estrogen.

Introduction of foreign genes into the mammary gland by adenoviral delivery

To determine the feasibility of introducing foreign genes into the mammary gland, we first injected indigo carmine as a vital tracking dye to examine the physical penetration of the dye throughout the mammary epithelial compartments (37, 40). Rats were anesthetized, and the main ducts were cannulated with a blunted 21–26 gauge needle. A single gland can accommodate injections of 2–40 μl of dye suspension depending on the age of the animal. Using this procedure, we were able to monitor the success of each injection and visualize the complete glandular structure within seconds after the injection (Fig. 3, A and B). The tracking dye diffused out of the mammary ducts completely within 20 h and became invisible. This dye was therefore used in combination with the adenovirus preparations to monitor the success of injection. In the first experiments, an adenoviral construct that contains the β-galactosidase gene under the control of the constitutive CMV promoter (Ad-CMV-βgal) in combination with the tracking dye was injected into the

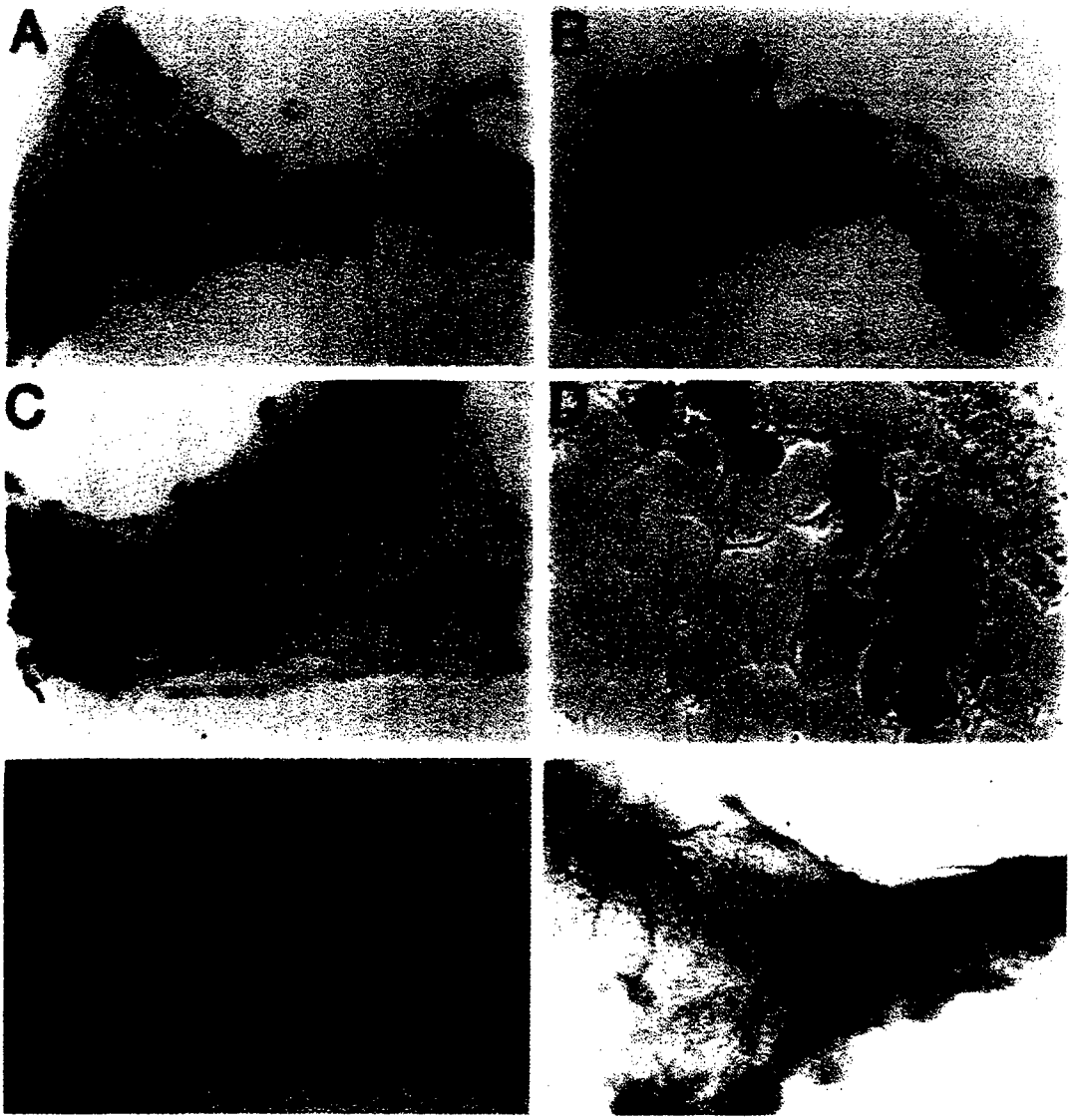

FIG. 4. Ad-CMV-βgal infection of rat mammary gland. Rats were anesthetized and mammary gland infused with 10 μl of tracking dye alone (A) or in combination with 4×10^7 pfu Ad-CMV-βgal (B–F). The dye diffused out in less than 24 h. Two days post intraductal injection, the mammary fat pads were dissected, fixed, and stained with X-gal solution. The blue cells represent the induction of β-galactosidase reporter activity. Panels A–C and E–F are whole mount staining, and panel D is the tissue section obtained from the gland stained in C. Panels E and F show that β-galactosidase expression (*blue color*) is diminished after 4 days (E) and is undetectable at 9 days after infusion (F). S, Stromal cells; LE, luminal epithelium; ME, myoepithelium.

mammary gland to examine its degree of infectivity in the mammary epithelium. To determine the conditions under which infection by the adenovirus is optimal, studies were performed to determine the appropriate titer of adenovirus to deliver to the gland and the appropriate length of time between administration of the virus and measurement of gene expression. Mammary glands were infused with varying amounts of the virus in conjunction with tracking dye and the mammary fat pads were dissected, fixed, and stained with X-gal at various time points after the injection. A multiplicity of infection of 10:1 was found to be sufficient to infect the epithelial cells with the assumption that the number of mammary epithelial cells per gland is approximately 3×10^7. The results in Fig. 4 demonstrate that while the tracking dye had completely disappeared at 48 h after injection of the dye alone (panel A), strong β-galactosidase expression was observed and persisted throughout the gland (panels B and C). Interestingly, analysis of this expression at high magnification revealed that the β-galactosidase staining was localized to the luminal epithelial compartment of the small ducts but not in the stromal compartments, indicating that the luminal epithelial cells preferentially take up the virus. The expression of β-galactosidase persisted but diminished dramatically 4 days after infusion of the virus (panel E) and was undetectable 9 days after infusion (panel F).

In situ localization of estrogen receptor-dependent gene expression

To reconstitute estrogen receptor-dependent reporter gene expression *in situ* in the mammary gland, we next tested the

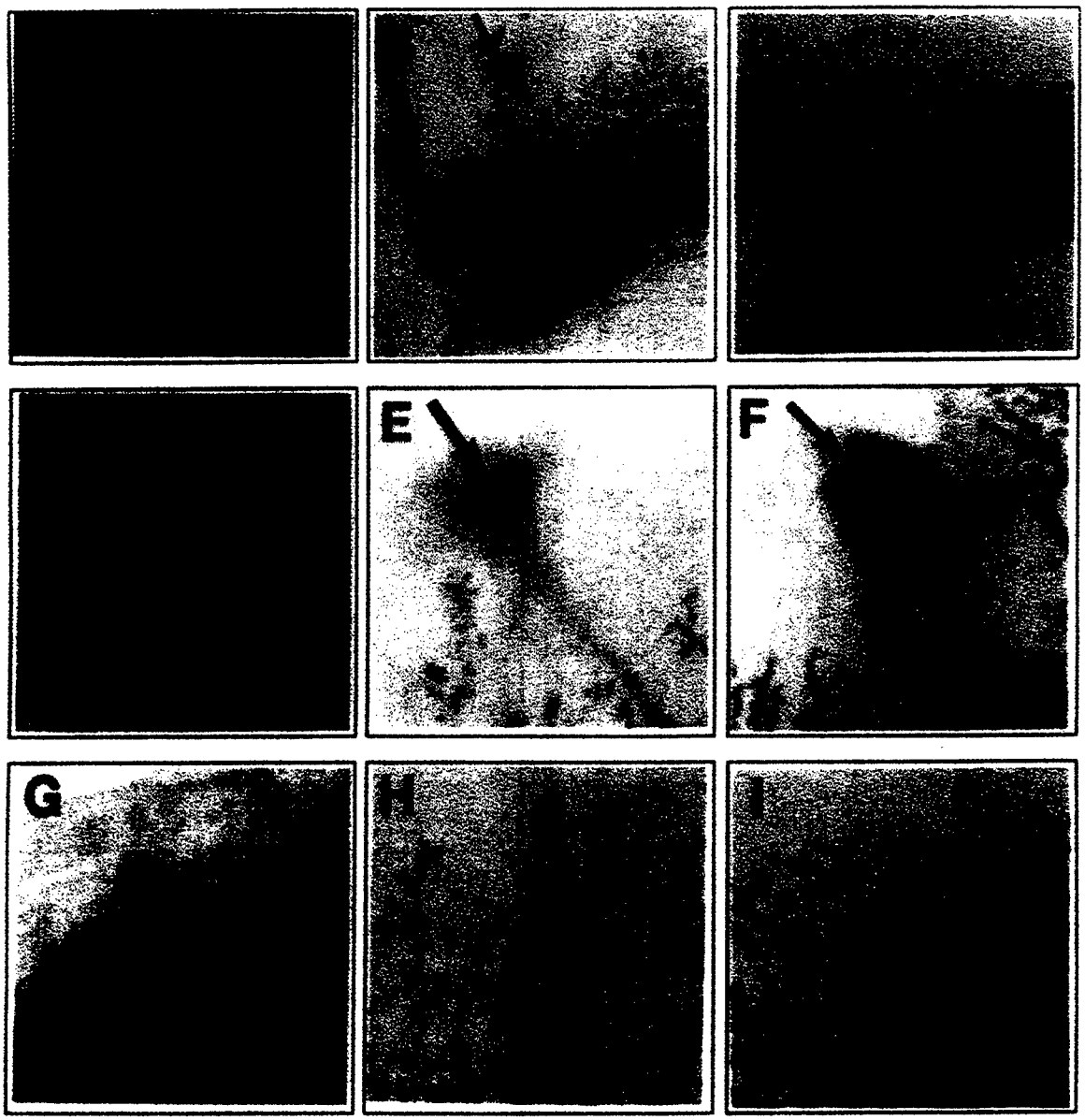

FIG. 5. Estrogen-dependent transcriptional activation of Ad-ERE-tk-βgal reporter gene in the mammary gland. Ovariectomized rats were anesthetized and the mammary gland infused with Ad-ERE-tk-βgal (1.6×10^8 pfu) (A and B) or Ad-CMV-βgal (4×10^7 pfu) (C) in conjunction with a tracking dye in a final volume of 10 μl. Rats receiving Ad-ERE-tk-βgal were injected with control vehicle sesame oil (A) or 100 μg estrogen benzoate (B) in a volume of 0.2 ml. The next day, mammary fat pads were dissected, fixed, and stained with X-gal. *Arrows* indicate the location of nipples that connect to the mammary main ducts and subsequently the lobulo-alveolar structures. High power magnification was also obtained to further illustrate the location of β-galactosidase at regions close to nipples (D–F) and at distal regions of the glands (G–I). D and G, High power magnification from panel A. E and H, Derived from panel B. F and I, Derived from panel C. *Blue* cells are the cells expressing β-galactosidase.

estrogen responsiveness of the two adenoviral reporter gene constructs we had generated. We injected glands with either the Ad-ERE-tk-βgal or Ad-ERE4-E1b-βgal reporter viruses together with the tracking dye. Rats were then injected with estradiol benzoate or control vehicle and the fat pads were removed 24 h later, fixed, and stained for β-galactosidase activity. Surprisingly, despite the comparable levels of induction of both reporter genes by estrogen when tested in MCF-7 cells, only the Ad-ERE-tk-βgal reporter gene responded to estrogen by expression of β-galactosidase when tested in the mammary gland, indicating that reporter gene expression was dependent on a strong promoter in the tissue. The estrogen responses obtained with the Ad-ERE-tk-βgal reporter virus are shown in Figs. 5 and 6. In the absence of estrogen (Fig. 5A and Fig. 6, A and B) very low basal expression of β-galactosidase was observed in the gland. However, in the mammary glands of rats treated with estradiol benzoate for 1 day, strong localized expression of β-galactosidase was detected (Fig. 5B and Fig. 6, C and D). The expression pattern was strikingly different from that observed using the constitutive β-galactosidase expression construct, Ad-CMV-βgal (Fig. 5C). Despite the ability of the

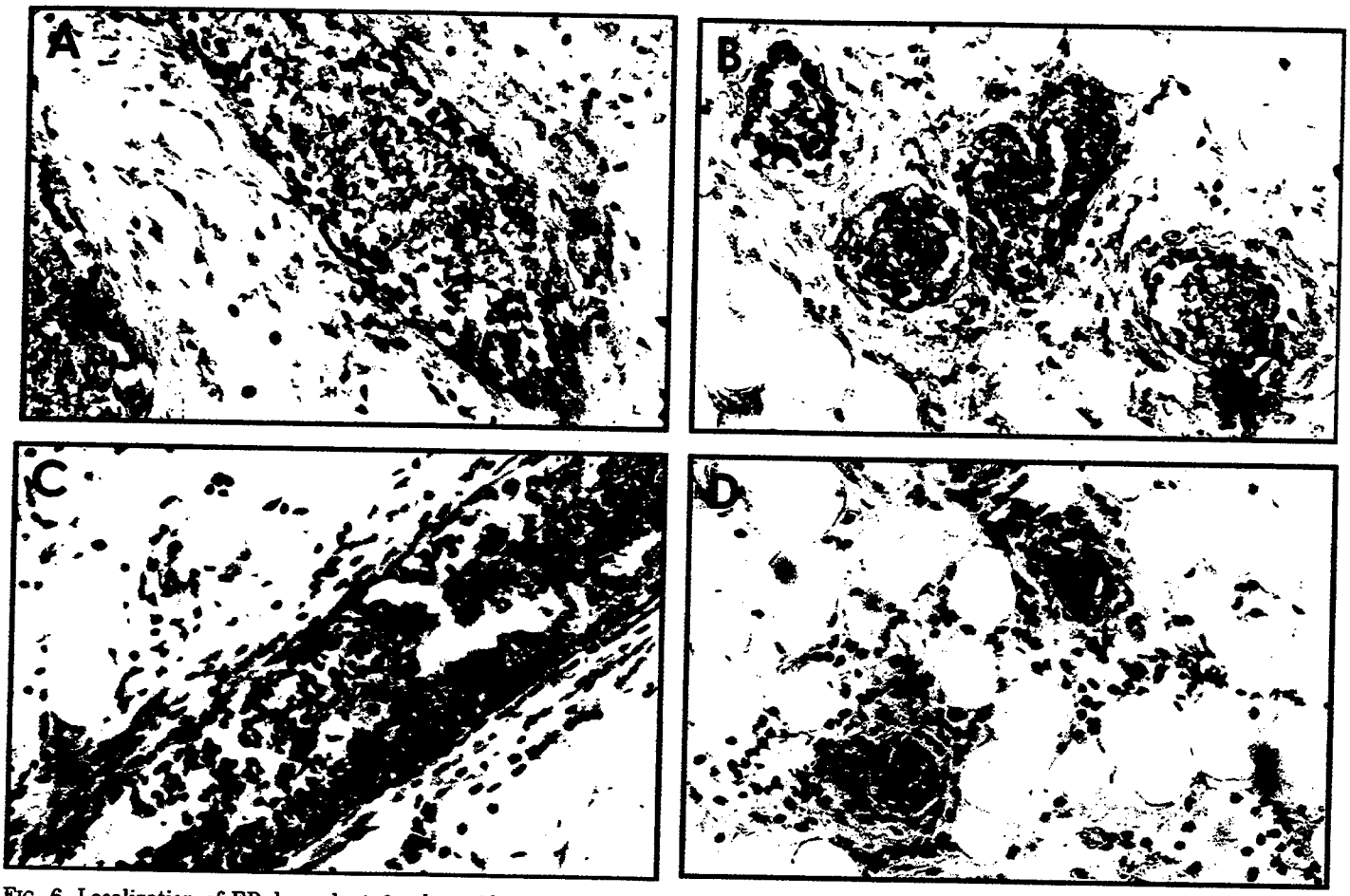

FIG. 6. Localization of ER-dependent β-galactosidase activity. Analysis of β-galactosidase stained sections (40× magnification) from control (panels A and B) and EB treated (panels C and D) rats showing large (A and C) and small (B and D) ducts. Sections were counterstained with nuclear fast *red*. *Blue* cells are the cells expressing β-galactosidase. Animals were treated the same way as mentioned in Fig. 5.

virus to penetrate epithelial cells located throughout the mammary ductal system as demonstrated using the CMV-βgal adenovirus (Fig. 5, C, F, and I), estrogen-dependent reporter gene expression was concentrated in the small ducts (Figs. 5H and 6D) as compared with regions close to nipples (Figs. 5E and 6C). These data indicate that the estrogen receptor activity is minimal in the large ductal epithelia at this time and is localized to the small ducts of the gland.

Localization of ER expression in mammary epithelium

The selective responsiveness of the small duct epithelium to estrogen suggested that either estrogen receptors are selectively expressed in the hormone responsive cells or that receptors in these cells are selectively more active in response to estrogen than those residing in the proximal ductal epithelium. To distinguish between these possibilities, we used an immunohistochemical approach to examine the expression of ER throughout the mammary gland. The results in Fig. 7 demonstrate that ER is expressed in both the epithelial and stromal cells with the most intense expression observed in the nuclei of the epithelium. These findings are similar to those previously obtained in adult mice using anti-ER antibodies and radioactive ligand binding assays (18, 41). Within the epithelial compartment, strong expression of ER was observed in numerous cell nuclei of the proximal large ducts (panel A) and more distal small ducts (panels B and C) with rare ER expression in the terminal end buds (panel D). In both large and small ductal epithelia, over 50% of the cells were positive for ER. These results clearly demonstrate that the lack of estrogen-dependent reporter gene expression in the proximal ductal epithelium is not due to a lack of estrogen receptor expression in this region but rather to a decreased sensitivity of these receptors to the hormonal stimulus.

Discussion

In this study, we have demonstrated the feasibility of introducing heterologous genes into the rat mammary gland *in vivo* using an adenoviral gene delivery approach. The adenovirus efficiently infected rat mammary epithelial cells throughout the mammary ductal system but did not reach the stromal compartment of the gland. Thus, it is most likely that the adenovirus cannot penetrate through multiple layers of the cells, especially the basal membrane that underlies the base of the mammary epithelial cells. Our results indicate that the adenoviral delivery strategy serves as a powerful tool to deliver specific genes to mammary epithelium, but its

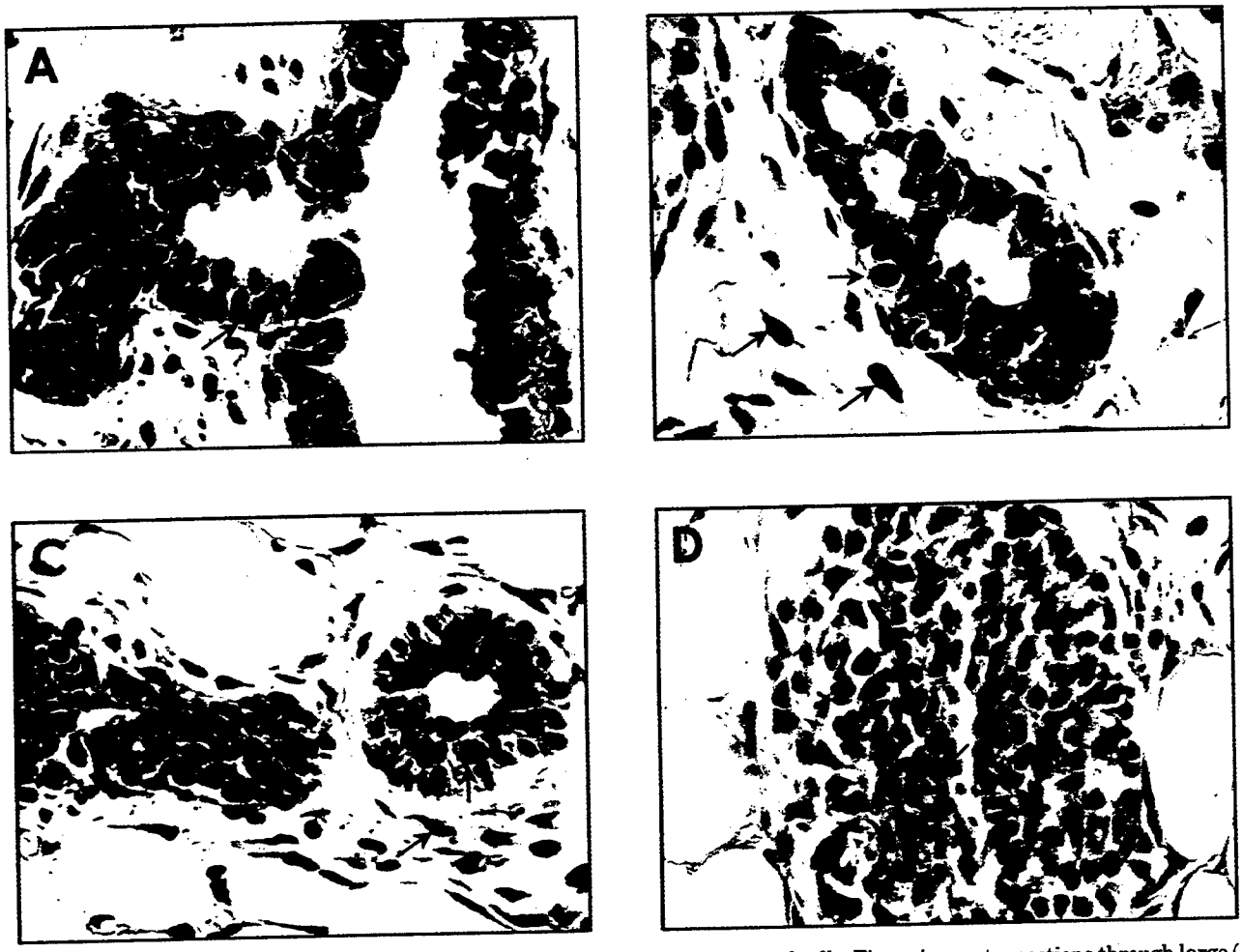

FIG. 7. Immunohistochemical localization of ERs in rat mammary epithelial and stromal cells. Five-micrometer sections through large (A) and small (B and C) ducts and end buds (D) probed with rabbit anti-ER IgG followed by peroxidase labeled secondary antibody and counterstained with hematoxylin (magnification, 40×). Animals were treated the same way as mentioned in Fig. 5.

utility may be restricted to epithelial cell targets. Nevertheless, the approach has several attractive features for monitoring and manipulating gene expression in the mammary gland. First, the virus can be purified at high titers allowing injection of minimal volume of material into the gland. Second, viral gene expression persists up to 4 days in the tissue, allowing ample time for experimental and hormonal manipulation of the animals and monitoring of transcriptional responses. Third, the ability of the virus to infect both proliferating and resting cells makes it ideally suited to localizing changes in hormone responsiveness that occur in proliferating and differentiated epithelial cells as a consequence of developmental stage, hormonal manipulation, or in response to carcinogen challenge.

Recombinant adenoviral vectors have been targeted to several cell lines *in vitro* and several organs *in vivo*. Adv-RSV-βgal has been used to infect several tissue culture cell lines like RKO, MDA-435, T47D, MCF-7, HT29, SHSY-5, SK-N-SH, IMR-32, K-562, and primary breast carcinoma cells (42) with greater than 95% cell efficiency. The adenovirus containing β-galactosidase gene alone demonstrated various degrees of toxicity in different cancer cell lines. The same group studied the functional role of bcl-x_s. adenovirus in the same cell lines and demonstrated the induction of apoptosis in over 90% of the cells. Recently, adenovirus-mediated overexpression of transcription factor E2F-1 has been shown to induce apoptosis in several human breast and ovarian carcinoma cell lines (43). Also, HC11 cells have been shown to be infected with high efficiency by an inactive LacZ adenoviral reporter and expression of LacZ achieved using Cre recombinase (44). The same expression has been reconstituted *in vivo* in transgenic mice expressing Cre-recombinase spatially and temporally under the control of the WAP or MMTV promoter. The inactive LacZ adenoviral vector was injected directly into the mammary gland, although it was not obvious if the delivery was by the intraductal route or into the adipose stroma. Reporter genes using adenoviral vectors have been delivered to several organs *in vivo* including salivary gland, lung, liver, gut, blood vessels, brain, CNS, chondrocytes, T cells, etc. The experiments reported herein extend previous studies by demonstrating the feasibility of intraductal injections in the normal mammary gland and

examining the functional role of an estrogen-dependent target gene.

Using this strategy, we have reconstituted localized estrogen-dependent activation of an exogenously introduced reporter gene in the mammary epithelium of the small ducts. In contrast to the constitutive reporter gene, the estrogen-dependent target gene response was concentrated at the level of the small ducts with very low activity detected in the large ductal epithelium. Thus, while the adenoviral vector can efficiently infect the large primary and secondary ductal epithelium, these cells appear to be refractory to the estrogen stimulus. Analysis of the expression of estrogen receptors throughout the gland demonstrated that lack of estrogen response is not due to a lack of estrogen receptor expression in large ductal epithelia. In contrast, this region of the gland is densely populated with estrogen receptors that appear to be relatively insensitive to the hormonal stimulus. These observations indicate that factors other than the ER that are necessary for the estrogen-dependent transactivation response are differentially expressed in estrogen sensitive epithelial cells.

Estrogen regulation of gene expression is known to be mediated by a hormone-dependent removal of corepressor proteins from the estrogen receptor and a stimulation of binding of coactivator proteins to the ER (25, 26). Binding of receptor coactivators enhances ER interaction with the general transcription apparatus and results in strong enhancement of the estrogen-dependent transactivation response. The central role of these coactivators in estrogen receptor activation suggests that the lack of ER activity in ductal epithelia may be due to a differential expression of estrogen receptor coactivators in subpopulations of mammary epithelial cells. In this regard, it will be of interest to determine which coactivators are coexpressed with estrogen receptors in the mammary epithelia and to evaluate how these proteins may contribute to the differential transcription regulatory responses of ER positive epithelial cells to the estrogen stimulus.

Acknowledgments

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DECREASED TRANSACTIVATION FUNCTION OF ESTROGEN RECEPTORS IN SITU DURING MAMMARY GLAND DEVELOPMENT. W.-S. Shim*, A. C. Eischeid, and M.-H. Jeng. Department of Medicine, University of Virginia Health Sciences Center, Charlottesville, VA. 22908.

Growth and differentiation of the mammary gland require estrogen receptor (ER)α as demonstrated in ERα knockout mice. Functional activation of ER mediated transcription during this process has not previously been studied. The transactivation function of the ERs can be regulated by estrogen, phosphorylation state of the receptors, multiple forms of receptor, the presence of co-activators and co-repressors, and the cellular context. We hypothesized that the expression of ERs and their functional activation may be differentially regulated during this process. To test this hypothesis, we infused an adenovirus construct containing β-galactosidase reporter gene under the control of estrogen response element and minimal promoter thymidine kinase (Ad-ERE-tk-βgal) into female rat mammary gland main ducts of various age groups and rats were then treated with estrogen benzoate. The next day, mammary fat pads were removed, stained with X-gal, sectioned, and then stained with various antibodies to determine the co-localization of transactivated blue cells, ERα protein, and viral product. Activation of ER mediated transcription would result in detection of β-galactosidase positive blue color. Ad-CMV-βgal and an anti-viral antibody were also used to determine the infection efficiency and expression of viral product in parallel sets of glands. In both cases, we detected high infection efficiency in mammary epithelial cells but not in stromal cells. When Ad-ERE-tk-βgal was infused into glands from different age groups, we found that the epithelial cells exhibited different sensitivity to estrogen in transactivating the reporter construct. It appeared that the ER transactivation was inversely correlated with the developmental age of the animals. This observation was not attributed to the diminished expression of ERα, nor the decreased infection efficiency because of the co-localization of viral 72 KD protein and ERα protein in all age groups. We also observed positional activation of ER and the activation predominantly occurred at small ducts/alveolar structures but not at large main ducts. Double fluorescent labeling using differentiation marker progesterone receptor and proliferating marker PCNA will be used to determine the relationship of ER functionality and differentiation stage of the gland. In summary, we have demonstrated a decreased ER transactivation function and a decreased sensitivity to estrogen in rat mammary epithelial cells in situ during mammary gland development. Our data suggest a decreased requirement of ER function during the process of aging and differentiation in mammary gland. Supported by Department of Defense Breast Cancer Research Program DAMD17-96-1-6233 and DAMD17-97-1-7066.

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DECREASED TRANSACTIVATION FUNCTION OF ESTROGEN RECEPTORS IN SITU DURING MAMMARY GLAND DEVELOPMENT.

Meei-Huey Jeng[1]*, Woo-Shin Shim[1] and Anne C. Eischeid[1]. [1]Department of Medicine, University of Virginia Health Sciences Center, Charlottesville, VA, 22908.

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DISCRETE EXPRESSION PATTERN OF STEROID RECEPTOR COACTIVATOR-1 (SRC-1) AND ESTROGEN RECEPTOR α (ERα) IN ESTROGEN-RESPONSIVE MAMMARY GLAND AND UTERUS.

W.-S. Shim*, R.J. Santen, M. Brown, and M.-H. Jeng. University of Virginia, Charlottesville, VA 22908 and Dana-Faber Cancer Institute, Boston, MA 02115

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FUNCTIONS OF STEROID RECEPTOR COACTIVATORS IN BREAST TISSUE. M.-H. Jeng*, W.-S. Shim, R.J. Santen, and C. Kao, M. Brown, M.-J. Tsai, B.W. O'Malley. Department of Medicine, Division of Hematology/Oncology and Department of Urology, University of Virginia Health Sciences Center, Charlottesville; Department of Adult Oncology, Dana-Farber Cancer Institute, Boston; and Department of Cell Biology, Baylor College of Medicine, Houston.

Estrogen receptor α (ERα) has been implicated in the development of both normal breast structure and breast cancer. The transcriptional activation of ERα can be enhanced by the steroid receptor coactivator-1 (SRC-1) family members such as SRC-1, GRIP1/TIF2/SRC-3 and p/CIP/AIB1/SRC-3. SRC-1 null mice display partial responses to hormone stimulation with subsequent impairment in branching, indicating the requirement of SRC-1 for full mammary gland development. Moreover, amplification of AIB1 in human breast and ovarian cancers indicated the involvement of SRC-1 family members in breast cancer formation. To better understand the physiological functions of SRC-1 family members, we examined the expression and the transactivation function of ERα in rat mammary gland using immunohistochemical staining, western blot analysis, and adenovirus delivery (to determine the *in situ* transcriptional activation of ERα target gene). We found that SRC-1 is segregated from ERα in normal mammary epithelium. Futhermore, the progesterone receptor (PR) was induced only in ERα-expressing epithelial cells, but not in SRC-1-expressing epithelial cells. In contrast, a vast majority of stromal cells coexpressed ERα and SRC-1. However, PR was not induced by estrogen in these stromal cells (PNAS, 96:208, 1999). While a vast majority of epithelial cells coexpressed ERα and GRIP1, p/CIP was also found to be coexpressed with ERα in these cells. Interestingly, GRIP1 and p/CIP were present in a majority of the mammary epithelial cells in SRC-1 null mice. When the *in situ* transactivation function of ERα was examined in normal mammary epithelium, we found that ERα-positive mammary epithelial cells displayed a differential sensitivity in a region-specific manner toward estrogen induction (Endocrinology, 139:2916, 1998). Our data suggest that SRC-1 is not necessary for ERα-mediated induction of PR in mammary epithelium and is also not sufficient for PR induction in stromal cells expressing both ERα and SRC-1. Our data raises the possibility that SRC-1 has cell type-specific functions rather than to act simply as a coactivator for ERα or PR and SRC-1 family members play important roles for the full development of mammary gland. We are currently examining the expression and hormonal regulation of SRC-1 family members in pregnant, lactating, and involuting rat mammary glands as well as in human breast tumors and in SRC-1 null mice. Overexpression of SRC-1 family members in normal breast using adenovirus delivery will allow us to determine the role of SRC-1 family members during normal breast development and cancer formation. Supported by Department of Defence Breast Cancer Research Program DAMD 17-96-1-6233 and DAMD 17-97-7066.

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M.-H. Jeng* and W.-S. Shim.
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STEROID RECEPTOR COACTIVATORS IN NORMAL MAMMARY GLAND

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Components of the estrogen signaling pathways have been shown to play important roles during normal mammary gland development and in breast cancer. This includes estrogen receptor α (ERα) and steroid receptor coactivator-1 (SRC-1) family members. SRC-1 family members can interact with ERα and enhance the ligand-dependent transcription. To better understand the physiological functions of SRC-1, we examined the expression of SRC-1 using immunohistochemical staining and Western blot techniques and the *in situ* ER transactivation function using adenoviral delivery system. We found that SRC-1 was segregated from ERα in immature and mature mammary epithelial cells. This finding was in contrast to the finding for the stroma, where significant numbers of cells coexpressed ERα and SRC-1. Treatment of animals with estrogen induced progesterone receptor (PR) expression in the ERα-expressing mammary epithelial cells in the absence of detectable SRC-1 and did not affect the segregated pattern of SRC-1 and ERα expression. PR was neither expressed nor induced by estrogen treatment in stroma, despite the coexpression of ERα and SRC-1. In addition, the *in situ* ER transactivation function in rat mammary gland was enhanced by estrogen treatment. We conclude that SRC-1 has cell type-specific functions other than simply to act as coactivator for ERα or PR in mammary epithelium and abnormal expression of coactivators may play a role in the growth stimulatory properties of estrogen in breast cancer.

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OVEREXPRESSION OF SRC-1 PROTEIN IN HUMAN BREAST TUMORS

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Steroid Receptor Coactivator-1 (SRC-1) is a member of the p160 steroid receptor coactivator family and interacts with steroid receptors to enhance ligand-dependent transcription. However, the functions of SRC-1 and its role in breast cancer have not been well clarified. Our previous studies demonstrated that SRC-1 was expressed in a subpopulation of rat mammary epithelial cells. Interestingly, SRC-1 was segregated from ERα in immature and mature mammary epithelium in virgin female rats. To further investigate the potential involvement of SRC-1 in breast epithelial proliferation and tumorigenesis, we examined the expression level of SRC-1 in human breast tumors and compared it to the expression level in adjacent normal breast tissue in 17 paired specimens using Western blot analysis and immunohistochemistry. β-actin level was used as a control for loading in Western blot analysis. Among 17 paired human breast specimens analyzed, we found that 5 of the breast tumors expressed a higher level of SRC-1 protein as compared to the normal adjacent breast tissue. After normalization with the β-actin level using a densitometer, the expression of SRC-1 protein level was found to be 2 to 4-fold higher in tumor specimens in these 5 cases as compared to the adjacent normal breast tissue. We also found that most of the tumor cells expressed SRC-1, and some of them showed stronger staining intensity. Furthermore, both ERα-positive and ERα-negative breast carcinomas expressed SRC-1 protein. Studies with more clinical specimens and an assessment of the relationship of SRC-1 overexpression with tumor differentiation, proliferation, and endocrine sensitivity are in the progress. In summary, our data suggest that breast tumors can arise from a subpopulation of breast epithelial cells and that overexpression of SRC-1 may play a critical role in breast cancer formation. Supported by Department of Defense Breast Cancer Research Program grants DAMD17-97-1-7066 and DAMD17-99-1-9430 to MHJ.

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Sponsor: Not Needed
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UP-REGULATION OF SRC-1 PROTEIN AND INCREASE OF ER IN SITU TRANSACTIVATION FUNCTION BY PREGNANCY HORMONES.

W.S. Shim[1], M.A. Turner[1], R.J. Santen[1] and M.H. Jeng[2]. [1]Department of Medicine, University of Virginia Health System, Charlottesville, VA, United States, [2]Department of Medicine, Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN, United States, [3]Department of Medicine, University of Virginia Health System, Charlottesville, United States

Steroid Receptor Coactivator-1 (SRC-1) has been demonstrated to be a coactivator for steroid, retinoid, and thyroid hormone receptors through direct ligand-dependent interaction with these receptors. We previously demonstrated that SRC-1 was segregated from estrogen receptor α (ERα) and progesterone receptor (PR) in virgin female rat mammary epithelium. To further investigate the roles of SRC-1 during mammary gland development, we examined the spatial and temporal expression of SRC-1 and the effects of pregnancy hormones on SRC-1 expression using Western blot analysis, Real Time RT-PCR, and immunohistochemistry. ER in situ transactivation function was also assessed by infusing the mammary ducts with an adenoviral vector expressing β-galactosidase reporter under the control of an estrogen response element (Ad-ERE-βgal). We found that SRC-1 was up-regulated and co-localized with ERα in mammary epithelium in pregnant female rats. Furthermore, SRC-1 remains segregated from ERα in mammary epithelial cells in involuting female rats. Treatment of ovariectomized rats with estrogen (E) alone was able to stimulate the Ad-ERE-βgal reporter activity, but not the segregation status of SRC-1 from ERα, in rat mammary epithelium. Progesterone (P) alone was not able to influence the expression of SRC-1, the segregation status of SRC-1 from ERα, or the ER transactivation function. In contrast, E + P treatment (to mimic pregnancy) was sufficient to stimulate the expression of SRC-1 protein and resulted in co-localization of SRC-1 with ERα in mammary epithelium. More importantly, E + P treatment greatly enhanced the Ad-ERE-βgal reporter activity in mammary gland. Our data suggest that up-regulation of SRC-1 is important in the ER transactivation function during pregnancy and that SRC-1 is involved in the full development of the mammary gland. Supported by DOD Breast Cancer Research Program DAMD17-97-1-7066 and DAMD17-99-1-9430 and RO1CA82565 (to M.-H.J.).

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Submitted

OVER-EXPRESSION OF SEVERAL NUCLEAR RECEPTOR COACTIVATOR PROTEINS IN HUMAN BREAST CARCINOMA.

Q.H. Zhang[1], L.Y. Chang[1], E. Vieth[1], M.R. Stallcup[2], D.P. Edwards[3], L. Cheng[1], R.J. Goulet[1] and M.H. Jeng[1]. [1]Depts ofMedicine, Surgery & Pathology, Walther Oncology Center,Indiana University School ofMedicine, Indianapolis, IN, United States, [2]Department of Pathology, University of Southern California, Los Angeles, CA, United States, [3]Department ofPathology, University of Colorado, Denver, United States

Coactivators ofnuclear receptors interact with steroid receptors to enhanceligand-dependent transcriptional activation. Although the roles of thesecoactivators havebeen intensively studied in in vitro systems, the expression of these coactivators has notyet beenwell characterized in human neoplasm. In this report, the protein expression of three estrogen receptor α (ER α) coactivators, Steroid Receptor Coactivator- 1 (SRC-1), Amplified In Breast Cancer (AIB1), andCoactivator Arginine Methyltransferase (CARM1), was analyzedin 40 cases of breast carcinoma and in 17 cases of paired adjacent normal breast tissue using Western blot analysis andimmunohistochemistry. β-actin level was used as a control for loading. We foundthat SRC-1, AIB1 and CARM 1 were expressed in many breast carcinoma specimens. Among the17 paired human breast specimens analyzed, 5 breast tumors expressed a higher level of SRC-1 protein and 4 casesexpressed a higher level of CARM1 protein, as comparedto thenormal adjacent breast tissue. After normalization with β- actin level by densitometer, the expression of SRC-1 and CARM1 protein level was found to be2 to 4-fold higher in tumor specimens. Immunohistochemical staining indicated that most of thetumor cells expressed SRC-1, AIB1, and CARM1, andmany of the tumor cells showed stronger staining intensity. Furthermore, both ERα-positive and ERα- negative breast cancer cells expressed SRC-1 andCARM1. The expression of SRC-1, AIB1 and CARM1 didnot correlate with the ERα expression. Studies with more clinical specimens and an assessment ofthe relationship of differential co-activator over- expression to clinicopathologicd characteristics are in progress. In summary, our datasuggest thatthe over- expression of SRC-1, AIB1, andCARM1 may play a role in breast cancer formation andprogression. Supported by DAMD17- 97-1-7066 and DAMD17-99-1- 9430 (to M.-H.J.).

FUNCTIONS OF ESTROGEN RECEPTOR COREGULATORS IN BREAST CANCER

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Estrogen signaling components have been shown to be important in breast cancer progression. These include estrogen receptor alpha (ER) and its coregulators such as steroid receptor coactivator 1 (SRC-1) family (p160) members, SRC-1, SRC-2/TIF2/GRIP1, and SRC-3/AIB1. These ER coregulators can regulate ER transactivation function in a ligand dependent fashion. Increasing evidence suggests that the cross-talk of steroid receptors with growth factor signaling may have an important role in regulating gene expression mediated by steroid receptors. This report examines the expression of SRC-1 family members in human breast tumors and the role of ER coregulators in cross-talk between growth factors and ER signaling. We analyzed 122 cases of breast carcinoma obtained from the IU Tissue Procurement Facility, 79 cases of breast carcinoma in tissue array slides obtained from NCI, and 29 cases of breast carcinoma in commercial breast sausage slides (containing an additional 15 cases of fibroadenoma, and 15 cases of normal breast tissue). We found that AIB1 protein was overexpressed (p-value < 0.001). When normal breast epithelial MCF10A cells and breast cancer MCF-7 cells were infected with an adenovirus expressing AIB1, the S phase was increased dramatically, suggesting that AIB1 was involved in cell cycle regulation. Furthermore, several growth factor signaling adapter proteins made up of Src homology (SH) domains, including c-Src, Grb2 and PLCg, interacted with ER in GST-pulldown assays. Interestingly, these same adapter proteins could also interact with SRC-1, GRIP1, and AIB1. The SH2 domain of c-Src was required for interaction with ER. The SH3 domain of c-Src was required for interaction with p160 coactivators. Interactions of c-Src with ER and p160 coactivators were further confirmed by co-immunoprecipitation. Protein kinase assay indicated that c-Src phosphorylated AIB1. The phosphorylation site was located at the C-terminal region of AIB1 containing the multiple LXXLL motifs important for interaction with steroid receptors. Our data suggest that coactivators can be the convergent point linking growth factor signaling to ER and that phosphorylation is likely a critical event for this type of gene regulation in breast cancer cells.